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The Role of T-box Proteins in Vertebrate Germ Layer Formation and Patterning

Sushma Teegala

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The Role of T-box Proteins in Vertebrate Germ Layer Formation and Patterning

Sushma Teegala

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
Abstract

All of the tissues in triploblastic organisms, with the exception of the germ cells, arise from the three germ layers, ectoderm, mesoderm and the endoderm. The identification of the genes that underlie the differentiation of these layers is crucial to our understanding of development. T-box family proteins are DNA-binding transcriptional regulators that play important roles during germ layer formation in the early vertebrate embryo. Well-characterized members of this family, including the transcriptional activators Brachyury and VegT, are essential for the proper formation of mesoderm and endoderm, respectively. To date, T-box proteins have not been shown to play a role in the promotion of the third primary germ layer, ectoderm. In this study, we have identified two T-box proteins, Tbx2 and Tbx3, as important players in the development of the vertebrate embryo with majority of our work focused on Tbx2. Our studies indicate that the T-box factor Tbx2 is both sufficient and necessary for ectodermal differentiation in the frog *Xenopus laevis*. Tbx2 is expressed zygotically in the presumptive ectoderm, during blastula and gastrula stages. Ectopic expression of Tbx2 represses mesoderm and endoderm, while loss of Tbx2 leads to inappropriate expression of mesoderm- and endoderm-specific genes in the region fated to give rise to ectoderm. Misexpression of Tbx2 also promotes neural tissue in animal cap explants, suggesting that Tbx2 plays a role in both the establishment of ectodermal fate and its dorsoventral patterning. Our studies demonstrate that Tbx2 functions as a transcriptional repressor during germ layer formation, and suggest that this activity is mediated in part through repression of target genes that are stimulated, in the mesendoderm, by activating T-box proteins.
In addition to Tbx2, we also identified Tbx3, another T-box transcription factor, whose expression begins early in development. Our data indicate that Tbx3 is expressed in the animal pole of *Xenopus laevis* embryos, where it functions to repress mesodermal and endodermal genes. Taken together, our results point to a critical role for T-box proteins in limiting the potency of blastula-stage progenitor cells during vertebrate germ layer differentiation.
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Table of Contents

Chapter I: Introduction

1.1 Xenopus laevis as a model organism ................................................................. Page 1
1.2 Fertilization to germ layer formation .............................................................. Page 4
1.3 Germ layer formation ....................................................................................... Page 6
1.4 The role of the TGFβ pathway in germ layer formation ................................. Page 7
1.5 Formation of ectoderm ..................................................................................... Page 9
1.6 Xema and its downstream targets ..................................................................... Page 9
1.7 Tbx2 and Tbx3 in development ........................................................................ Page 10

Chapter II: Materials and Methods .................................................................. Page 15

Chapter III: Tbx2 is required for the suppression of mesendoderm during early Xenopus development

3.1 Background ....................................................................................................... Page 22
3.2 Results .............................................................................................................. Page 25
   • 3.2.1 Expression of Xenopus tbx2 ................................................................. Page 25
   • 3.2.2 Tbx2 inhibits mesendoderm gene expression ....................................... Page 26
   • 3.2.3 Tbx2 promotes neural fate .................................................................... Page 30
   • 3.2.4 Tbx2 is required for the suppression of mesendoderm in the animal pole Page 31
   • 3.2.5 Tbx2 functions as a repressor .............................................................. Page 33
   • 3.2.6 Tbx2 represses direct targets of transactivating T-box proteins .......... Page 35
3.2.7 Repression of \textit{bix4} by Tbx2 is mediated by T-box binding sites on the \textit{bix4} promoter.

3.3 Discussion and Future directions

Chapter IV: Characterizing mediators, regulators and identifying functional domains of Tbx2

4.1 Background

4.2 Results

- 4.2.1 Tbx2 represses cycloheximide-induced genes.
- 4.2.2 Tbx2 strongly represses \textit{goosecoid} –\textit{LUC}.
- 4.2.3 BMP signaling may be crucial for inducing the expression of \textit{tbx2}.
- 4.2.4 Identifying functional domains of Tbx2.

4.3 Discussion and Future Directions

Chapter V: Tbx3 may play a role in specification of ectoderm

5.1 Background

5.2 Results

- 5.2.1 Xema is necessary but not sufficient for the expression of \textit{tbx3}.
- 5.2.2 Spatio-Temporal expression of \textit{tbx3}.
- 5.2.3 Activity of \textit{Xenopus laevis tbx3}.

5.3 Discussion and Future Directions

Chapter VI: Conclusion
List of Illustrations

Chapter I:

Figure 1.1: The *Xenopus laevis* life cycle ......................................................... Page 3

Figure 1.2: Diagram depicting the animal cap assay................................. Page 3

Figure 1.3: Fate map of *Xenopus laevis*......................................................... Page 5

Chapter III:

Figure 3.1: *tbx2* expression in the blastula and gastrula stage ectoderm is dependent on Xema activity................................................................. Page 27

Figure 3.2: Ectopic Tbx2 suppresses mesendoderm induction................ Page 29

Figure 3.3: Tbx2 promotes neural fate. .......................................................... Page 32

Figure 3.4: Tbx2 knockdown leads to ectopic expression of mesendodermal marker genes................................................................. Page 34

Figure 3.5: Tbx2 functions as a repressor during germ layer differentiation................................................................. Page 36

Figure 3.6: Tbx2 represses downstream targets of *VegT* and *Brachyury*................................. Page 39

Figure 3.7: A model of Tbx2-mediated ectodermal specification................ Page 42
Chapter IV:

Figure 4.1: Tbx2 represses cycloheximide-mediated induction of the mesodermal genes goosecoid and brachyury………………………………………………………………………………………… Page 47

Figure 4.2: Tbx2 represses gsc-luciferase………………………………………………………………………………………………………………………… Page 50

Figure 4.3: Loss of BMP signaling leads to loss of tbx2 expression………… Page 54

Figure 4.4: Tbx2 deletion constructs identify distinct functional domains……… Page 56

Chapter V:

Figure 5.1: tbx3 expression is lost upon Xema knockdown but is not induced by Xema………………………………………………………………………………………………………………………………………………………………………………………… Page 62

Figure 5.2: tbx3 is expressed in the animal cap during blastula stages………… Page 64

Figure 5.3: Ectopic tbx3 represses mesoderm and endoderm induction………… Page 66
Chapter I: Introduction

One of the amazing wonders of biology is realizing that the formation of a complex multicellular organism originated from one single cell - the fertilized egg. In metazoan organisms, the development of a multicellular organism begins with a single cell that divides mitotically to produce all the cells, tissues and organs of the body. But, what are the instructions that a single cell transmits from one generation to the next during the formation of an organism? How does that single cell give rise to an adult body with hundreds of different types of cells, that are organized into intricate tissues and organs? How are the organs arranged in such a way that our fingers develop at the tips of our hands and not elsewhere? These are some of the questions that developmental biologists attempt to understand and address by identifying the mechanisms that underlie the building of an organism.

1.1: Xenopus laevis as a model organism:

While a wide and diverse group of model organisms, including *C. elegans, Drosophila, Danio rerio*, and *Mus musculus* are used to address different aspects of development, the frog, *Xenopus laevis*, has been an invaluable model organism for the study of early development. It has been used to answer many questions regarding the molecular and genetic aspects underlying early embryonic development. It is relatively easy to obtain eggs from *Xenopus* as the females can be induced to produce eggs year-round upon injection of human chorionic gonadotropin hormone. Female frogs lay about 500 – 3000+ eggs, which can easily be fertilized *in vitro* with sperm obtained from the testes of a male frog. The resulting zygote, visible to the naked eye, is hardy,
fully aquatic, and easy to maintain in the laboratory. Most importantly, the embryos can be manipulated by microdissection, and exogenous nucleic acid and protein can be introduced via microinjection. The cells of the early embryo undergo rapid cleavage; these cells will eventually differentiate and give rise to the different tissues and organs of the adult. One of the stages during this period of early division is called the blastula stage, which is about 2000 - 4000 cells in the embryo and can be observed approximately three hours after fertilization (Figure. 1.1). The blastula stage – during which the embryo is comprised of a hollow ball of pluripotent cells--provides an important tool for developmental biologists as it allows for a simple, quick and inexpensive bioassay called the animal cap assay. The animal cap is the name given to the polar region of the animal hemisphere of a *Xenopus* embryo; once excised, the cap can be used for a variety of assays including the analysis of misexpression of RNA transcripts and proteins, inductive interactions, competence and morphogen gradients. Animal caps can be induced to form different types of tissue upon exposure to inductive signals, which include growth factors found in the early embryo. For example, excised animal caps if left in simple saline solution give rise to epidermis, an ectodermal derivative; however, exposure to the transforming growth factor-β (TGF-β) protein Activin, can induce both mesoderm and endoderm in a dose dependent manner (Sive, 1993). For this reason, scientists can study novel genes, and their function in germ layer patterning, in part by observing their effects in an animal cap assay (Figure. 1.2). Thus, animal cap assays provide a valuable tool with which one can study the complex mechanisms involved in patterning the embryo.
**Figure. 1.1: The *Xenopus laevis* life cycle.** This diagram represents the life cycle of *Xenopus laevis*. (Adapted from Xenbase.org)

**Figure. 1.2: Diagram depicting the animal cap assay.** Tissue excised from the animal pole at blastula stages forms epidermis in the absence of exogenous inducers; it can differentiate into neural, mesodermal, or endodermal tissue upon exposure to inducers (Ariizumi et al. Current protocols on stem cell biology Unit1, 2009).
1.2: Fertilization to germ layer formation:

The mature *Xenopus* egg has a distinct polarity, with a dark, pigmented animal region, and a pale and yolky vegetal region. The fertilized embryo undergoes rapid programmed mitotic divisions and relies entirely on maternally provided stores of proteins and mRNA until the “mid-blastula transition” (MBT), when the cell cycle lengthens due to introduction of the G1 and G2 gap phases. At this point, the embryo switches from relying on maternal stored mRNAs to initiating transcription of its own genome. The cells of the blastula are three tiered with the animal pole at the top, the equatorial or “marginal zone” in the middle, and the vegetal pole at the bottom; these regions will give rise to the germ layers, ectoderm, mesoderm and the endoderm, respectively. The three germ layers will eventually give rise to all of the tissues in the organism, with the exception of the germ cells: the ectoderm will give rise to the skin, neural crest, and central nervous system, the mesoderm to the tissues which include the blood, kidneys, notochord and somites, and the endoderm to the tissues including the liver, pancreas and the lining of the gut (Figure. 1.3). The accurate formation of the germ layers is critical as they are the foundation from which all tissues, organs and eventually the organism itself develops. In order to understand the process by which a fertilized egg becomes an organism, it is essential to understand the signaling pathways and gene regulatory networks that underlie germ layer formation.
Figure 1.3: Fate map of *Xenopus laevis*. The fate map depicts the organization of the germ layers at late blastula stages; the ectoderm (blue) derives from cells in the animal cap region, the endoderm (yellow) derives from cells in the vegetal pole. The mesoderm is induced in the “marginal zone” bordering the ectoderm and the endoderm. Some of the tissues that arise from these layers is also indicated. (Adapted from Richard Harland and John Gerhart (1997). Formation and Function of Spemann’s organizer.)
1.3: Germ layer formation:

In the *Xenopus* blastula, the fate of individual cells can be followed by injecting cells with a lipophilic dye and observing the location of the labeled cells later in development. The fate map of the blastula stage embryo reveals that the ectoderm arises from the animal hemisphere, the mesoderm from the marginal zone cells located in between the animal and vegetal hemisphere, and the endoderm emerges from the yolky vegetal hemisphere. This can be observed by culturing explants from the early blastula. Vegetal pole explants from the bottom layer have the potential to form endoderm when cultured and allowed to develop *in vitro*. This is due to the vegetally localized maternal T-box transcription factor, Veg-T, that is essential for the initiation of endoderm formation (Horb and Thomsen, 1997; Stennard et al., 1996; Zhang and King, 1996). Veg-T is required for the subsequent activation of Nodal related genes of the TGF-β (Transforming Growth Factor-β) family of ligands and other endoderm genes required for the maintenance of the endoderm (Shivdasani, 2002; Zhang et al., 1998). Signals from the endoderm also act on the overlying cells to induce the formation of mesoderm. The inducing factors secreted from the underlying endoderm were later identified as Nodal and Activin ligands of the TGF-β family (Kimelman and Kirschner, 1987). This can be observed *in vitro*; when explants from the animal and vegetal region are cultured separately, they give rise to ectoderm and endoderm, respectively. However, when the explants are combined, mesoderm is induced in cells from the animal region (Dale and Slack, 1987). The confirmation that the animal cap cells, and not the vegetal cells, form mesoderm was obtained by pre-labeling the animal region of the blastula with a cell lineage marker and showing that the labeled cells that formed the
mesodermal derivatives muscle, notochord and mesenchyme came from the animal cap tissue (Nieuwkoop, 1969). Thus, it is clear that the vegetal region induces the equatorial cells in the overlying animal region to form the mesoderm. The formation of ectoderm, however, is not thought to be an inductive event, and it has been generally considered that cells not under the influence of the VegT pathway follow a default pathway and differentiate as ectoderm (Zhang et al., 1998). This can be observed in explants, where an intact animal cap explant isolated from the blastula stage embryos give rise to ectoderm in the absence of external signals (Holtfreter and Hamburger 1955; Nieuwkoop 1969). The ectoderm itself gives rise to several different fates, most prominently neural and epidermal tissue; intact ectodermal explants normally express BMP4 and develop into epidermis while dissociation of the ectodermal cells dilutes the concentration of BMP essentially to zero, unveiling the “default” neural fate of these cells (Munoz-Sanjuan and Brivanlou, 2002). There is evidence that neural induction involves suppression of induction of the epidermal fate; thus, the default stage of the naïve ectoderm can be considered neural and not epidermal (Munoz-Sanjuan and Brivanlou, 2002).

1.4: The role of the TGFβ pathway in germ layer formation:

Secreted factors of the TGFβ superfamily play critical roles in regulation of the embryonic germ layers. The TGF-β superfamily is comprised of many ligands, including Activin, Nodal, Bone Morphogenetic Proteins (BMPs), and Growth and Differentiation factors (GDFs) (Grönroos et al., 2012). In general, signaling is initiated upon ligand binding to cell-surface transmembrane serine/threonine kinase receptors. Upon binding to the ligand, the activated receptor transduces
the signal to the nucleus through a family of intracellular signaling proteins called the R-Smads. The R-Smads include Smad 2 and Smad 3 for the TGF-β/Activin/Nodal pathway, or Smad 1,5,8 for the BMP pathway; the R-Smads form a complex with the common mediator, Smad 4, which translocates to the nucleus to regulate the transcription of target genes (Wrana et al., 1994). In the vertebrate embryo, Smads 1/5/8, mediators of BMP signaling, induce ventral mesoderm, while Smad2, activated by Activin, induces endoderm, dorsal, or ventral mesoderm in a concentration dependent manner (Ariizumi et al., 1991; Sive, 1993; De Robertis and Kuroda, 2004). Expression of dominant inhibitors of Activin-like signals results in disruption of mesoderm and endodermal specification (Chang et al., 2000). Similar mechanisms underlie the formation of mesoderm and endoderm and therefore we call it mesendoderm field in the early embryos, as it contains precursors to both mesodermal and endodermal cells. BMP ligands can induce prospective ectoderm to form ventral mesoderm, ventralize dorsal mesoderm and suppress neuralization in favor of ventral ectoderm. Elimination of BMP signaling by overexpressing a dominant negative BMP receptor, neuralizes animal caps, suggesting that BMP signaling is required for ventralization of the embryo (Faure et al., 2000). Besides these inductive signals that induce germ layer specification there are additional mechanisms that ensure proper germ layer differentiation. Studies from our lab and others have described the presence of signals that act to suppress inappropriate germ layer formation (Suri et al., 2005; Mir et al., 2007). These studies indicate that an additional mechanism of control prevents cells in a particular germ layer from differentiating into a cell type characteristic of another layer.
1.5: Formation of ectoderm

In the early *Xenopus* embryo, active restriction of inappropriate germ layer formation plays a critical role in the differentiation of the ectoderm. Maternal factors including Coco, Ectodermin, SRF, and the zygotic factor XFDL56 repress mesoderm and endoderm in the early embryo via distinct mechanisms, thus allowing for proper ectodermal development (Bell et al., 2003; Dupont et al., 2005; Sasai et al., 2008; Yun et al., 2007). Studies in our lab and by others have demonstrated that misexpression of the Fox family DNA-binding protein Xema/Foxi1e stimulates ectodermal differentiation in cells fated to give rise to mesoderm, while Xema knockdown leads to the ectopic formation of mesoderm and endoderm in the embryonic ectodermal field (Mir et al., 2007; Suri et al., 2005). These studies again point to a requirement for suppression of mesodermal and/or endodermal (mesendodermal) cell fate during ectodermal differentiation. However, Xema functions as a transcriptional activator, suggesting it likely mediates mesendoderm suppression indirectly, via activation of one or more transcriptional targets (Suri et al., 2005). A prominent role for transcriptional repression during ectodermal differentiation has not previously been demonstrated.

1.6: Xema and its downstream targets:

In order to identify transcriptional targets of the transcriptional activator, Xema, we performed a microarray screen as described in (Sridharan et al., 2012). RNA from animal cap explants were extracted from embryos injected with Xema or Xema morpholino to generate hybridization probes for use on *Xenopus laevis* Genome Arrays (Suri et al., 2005). The microarray screen
identified two T-box proteins, Tbx2 and Tbx3 as downstream targets whose expression decreased in Xema morpholino injected caps when compared to uninjected or control scrambled morpholino injected samples.

1.7: Tbx2 and Tbx3 in development:

Tbx2 and Tbx3 are proteins that belong to the T-box family, whose members are defined by a highly conserved DNA-binding motif, called the T-box domain (Abrahams et al., 2010). The T-box gene family is ancient: based on phylogenetic analysis, it is thought to have a common ancestor that underwent genome wide-duplication over 600 million years ago, during the early evolution of vertebrates (Agulnik et al., 1996). Currently, the T-box family can be divided into five subfamilies, namely T, Tbx1, Tbx2, Tbx6 and Tbr1 (Papaioannou and Silver, 1998).

*Brachyury*, a prominent and well-studied T-box gene, is a member of the T subfamily, whose role has been well established in the field of developmental biology. In mice, homozygous mutations in *brachyury* lead to embryonic lethality and a loss of the posterior mesoderm (Naiche et al., 2005). In addition, *brachyury* has been shown to play prominent roles during many stages of development in many organisms. Since the discovery of Brachyury, many laboratories have focused on identifying, cloning and determining the spatiotemporal localization and function of other members in the T-box family. Both induced and spontaneous mutations in T-box genes have revealed the importance of T-box genes in a wide range of tissues and organs during development. Importantly, mutations in T-box genes have been shown to contribute to several human syndromes (Naiche et al., 2005). Mutations in *Tbx1* gene have been implicated in
DiGeorge syndrome, a disorder characterized by craniofacial, glandular, vascular, and heart abnormalities in humans (Baldini, 2003), and mutations in Tbx5 is associated with Holt-Oram syndrome, characterized by abnormal bones in the upper limbs along with congenital heart defects (Li et al., 1997); while mutations in Tbx22 have been shown to cause X-linked cleft palate with ankyloglossia, a congenital oral anomaly (Braybrook et al., 2001). Additionally, human Tbx20 mutations were associated with atrial septal defects and cardiomyopathy; Tbx20 null mice die at mid-gestation due to the lack of proper heart chamber formation and cardiac hypoplasia (Singh et al., 2005; Stennard et al., 2005). In zebrafish, Morpholino-mediated knock-down of Tbx20 was found to cause defective heart chamber morphology and impaired development of the atrio-ventricular boundary in embryonic hearts (Szeto et al., 2002). The existence of congenital syndromes in humans and developmental defects in other organisms caused by disruption of Tbx genes, demonstrate their importance, making them important candidates to study and analyze for their roles in development.

In our screens, we identified two T-box genes that are downstream targets of Xema, Tbx2 and Tbx3. Tbx2 and Tbx3 are members of the Tbx2 subfamily, whose members also include Tbx4 and Tbx5 (Agulnik et al., 1996; Bollag et al., 1994). Tbx4 and Tbx5 have important roles in specifying limb identity and development. Tbx4 and Tbx5, when misexpressed in chick embryos, led to induction of additional limb and leg like structures, while dominant-negative forms of these genes in the prospective limb fields produced limbless phenotypes (Takeuchi et al., 1999). In addition, Tbx4 and Tbx5 are expressed in both lung and tracheal mesenchyme (Chapman et al., 1996); homozygous mutants of Tbx4 and Tbx5 die at E10 due to defects in lung and heart
development (Bruneau et al., 2001). The other members of this subfamily, Tbx2 and Tbx3 also play important roles; mutations leading to partial duplication of the human Tbx2 locus, 17q23.1q23.2 have been implicated in numerous abnormalities, including birth defects in the formation of the palate, congenital heart disease, craniofacial defects, and neuromuscular problems (Ballif et al., 2010). Ulnar-mammary syndrome, a cutaneous condition characterized by nipple and breast hypoplasia is caused by mutations that affect the DNA binding domain of Tbx3 (Bamshad et al., 1999). Other mutational studies in mice have also revealed that the homozygous inactivation of either Tbx2 or Tbx3 results in embryonic lethality (Davenport et al., 2003; Harrelson et al., 2004). Tbx2 and Tbx3 have also been implicated in cell cycle regulation and in the genesis of several cancers. In one study, researchers found overexpression of both genes in mammary gland neoplasia and breast cancer cell lines, suggesting a role for Tbx2 and Tbx3 in tumorigenesis and cancer progression (Douglas and Papaioannou, 2013; Peres et al., 2010). Tbx2 and Tbx3 are required for the regulation of cell proliferation during heart remodeling in the development of the heart myocardium in chick (Ribeiro et al., 2007). In the chick, Tbx2 and Tbx3 function as transcriptional repressors of the chamber genetic program in the non-chamber myocardium allowing for the proper development of the heart (Carreira et al., 1998; He et al., 1999; Ribeiro et al., 2007). Additionally, TBX-2 is the sole member of the Tbx2 subfamily that is expressed in C. elegans, where it is necessary for the formation of anterior pharyngeal muscles (Huber et al., 2013; Smith and Mango, 2007). Despite the essential role that the T-box proteins, Tbx2 and Tbx3 play in a wide variety of developmental processes, the pathway by which they function is still unclear.
In this dissertation, we first report the function of the T-box family factor Tbx2 during ectodermal development. tbx2 is expressed in the presumptive ectoderm at blastula and gastrula stages. Misexpression of Tbx2 inhibits differentiation of both ventral ectoderm and growth factor-induced mesendoderm, and promotes neuralization, the latter via repression of Bone Morphogenetic Protein (BMP) pathway activity. Tbx2 functions as a transcriptional repressor, and appears to regulate a set of target genes that overlap with those regulated by transactivating T-box proteins, including Xbrachyury and VegT. Finally, Tbx2 knockdown promotes ectopic mesendoderm differentiation; taken together, these studies demonstrate that Tbx2 is sufficient and necessary for the transcriptional suppression of inappropriate germ layer formation in the presumptive ectoderm. In chapter IV, we identify and characterize novel targets of Tbx2; in addition, we provide evidence to suggest that the BMP pathway may regulate the expression of Tbx2. We also describe the construction of deletion constructs of Tbx2 and present data suggesting that the C-terminal and N-terminal domains of Tbx2 are both important for repressing ventral ectodermal genes.

Finally, in Chapter V, we report the characterization of Tbx3, another T-box protein whose expression is lost in Xema morpholino-injected embryos. tbx3, like tbx2, is expressed during early developmental stages in the animal hemisphere. Misexpression of Tbx3 seems to also repress mesodermal and endodermal genes. However, while Tbx2 is a potent repressor of all mesodermal and endodermal genes, the function of Tbx3 seems to preferentially repress endodermal and ventral mesodermal, but not dorsal mesodermal genes. These results point to
crucial and partially non-overlapping roles for repressor T-box proteins during early germ layer development.
Chapter II: Material and Methods

Gene chip analysis and isolation of Tbx2 and Tbx3

Gene chip analysis was performed as described in Sridharan et al., 2012. Briefly, RNA from 80 animal cap explants, derived from blastula stage embryos injected with 1ng Xema or b-galactosidase RNA, 63ng 1:2 Xema MO1: Xema MO2, or 62.5ng scrambled morpholino (CMO) and cultured to stage 11, were used to generate hybridization probes for use on Affymetrix GeneChip Xenopus laevis Genome Arrays (Suri et al., 2005). Microarray data were normalized by RMA (Irizarry et al., 2003) and analyzed using the affylmGUI Bioconductor package (Wettenhall et al., 2006).

Xenopus tbx2 and tbx3 were isolated in a microarray screen to identify transcriptional targets of Xema (Suri et al., 2005) (Sridharan et al., 2012). The probe sets (XI.931.1) corresponding to tbx2 and probe set (XI.975.1. S1_at) corresponding to tbx3 were down-regulated in Xema morpholino-injected animal pole ectodermal explants at stage 11, when compared to uninjected or control scrambled morpholino-injected samples, respectively. A full-length tbx2 cDNA was a gift from the lab of Jin Kwan Han (Cho et al., 2011). Xenopus tbx3 was purchased from Open Biosystems and cloned into the CS++ vector using the ClaI and XbaI restriction sites.

Preparation of Tbx2 repressor and activator fusion constructs

For the Tbx2-DBD-VP16 and Tbx2-DBD- EnR constructs, residues 410-490 of the VP16 activator (Kessler, 1997) and residues 1-298 of the Drosophila Engrailed repressor (Kessler,
1997), respectively, were cloned downstream of the Tbx2 DNA-binding domain (residues 91-279).

**Preparation of Deletion constructs**

\[ Tbx2-\Delta C-F: 5’GGATCCATGAGAGATCCAGCTTTTC-3’ \]
\[ Tbx2-\Delta C-R: 5’CTCGAGTCAAAGGCTGCCATTATCCAG-3’ \]

\[ Tbx2-\Delta C-F: 5’GGATCCATGAGAGATCCAGCTTTTC-3’ \]
\[ Tbx2-\Delta C-R: 5’CTCGAGTCAAAGGCTGCCATTATCCAG-3’ \]

\[ Tbx2-\Delta N-FL-F: 5’GGATCCCCATGGACGCTAGTCTGGGCTGGAGC-3’ \]
\[ Tbx2-\Delta N-FL-R: 5’CTCGAGTCACCTTTGGGTGTCTCCTAAATCC-3’ \]

\[ Tbx2-\Delta N-FL-F: 5’GGATCCCCATGGACGCTAGTCTGGGCTGGAGC-3’ \]
\[ Tbx2-\Delta N-FL-R: 5’CTCGAGTCACCTTTGGGTGTCTCCTAAATCC-3’ \]

The above primers were used to amplify truncated versions of Tbx2 and were cloned into CS2++ vector using the BamHI and XhoI restriction sites.

**RNA preparation, explant dissection, and cell culture**

RNA was synthesized *in vitro* in the presence of cap analog using the mMessage mMachine kit (Ambion). Microinjection, explant dissection, and cell culture were performed as described (Hemmati-Brivanlou and Melton, 1994; Wilson and Hemmati-Brivanlou, 1995).
Luciferase assays

The BMP-responsive, Wnt-unresponsive Vent-2 promoter (TCFm-Luc) was a gift from the S. Sokol laboratory (Hikasa et al., 2010). Mutation in the putative T-box Binding Element (TBE) (GGGTGA to GGGGTC) was generated by GENEWIZ and independently sequenced. Embryos were injected at the 2-cell stage with 50 pg of TCFm-Luc reporter plasmid, with or without tbx2 RNA, (Hikasa et al., 2010); 5 pg of Renilla luciferase reporter was co-injected with all samples as an internal control. Samples of five embryos each were collected in triplicate at stage 11 for analysis.

The Bix4-Luc promoter was a gift from Elena M Silva (Casey et al., 1999; Tada et al., 1998). The Td construct was generated by GENEWIZ and independently sequenced. The mutations in Td-Luc, were introduced as described in (Casey et al., 1999). The triple mutant, Tdmp-Luc, in which all three characterized T-elements in bix4 were mutated, was generated by GENEWIZ and independently sequenced. Tm and Tp mutations were introduced as described in Casey et al. (1999). Samples of five embryos each were collected in triplicate at stage 11 for analysis.

The goosecoid-Luc promoters, -1500bp and -226bp, were gifts from Ken Cho (Addgene plasmid # 17159 and Add gene plasmid # 17158). The -492 goosecoid-Luc and TM123-goosecoid-Luc were gifts from Norihiro Sudou (Sudou et al., 2012). For all goosecoid-Luc experiments, animal caps were dissected from injected embryos at stage 8 and cultured for 3 hours in the presence or absence of activin, followed by luciferase assays.
For all Luciferase assays, embryos or animal caps were lysed in 200ul passive lysis buffer (Promega); 20 µl was assayed for luminescence.

**Whole mount *in situ* hybridization**

Whole mount *in situ* hybridization was carried out using standard protocols (Harland, 1991). BM Purple (Roche) was used for chromogenic reactions.

**Morpholinos**

Morpholino antisense oligonucleotides (GeneTools LLC) were designed to hybridize to the 5’ region of *tbx2* mRNA to block translation. Morpholinos were heated at 65°C for 5 minutes, and then cooled on ice prior to microinjection. The Tbx2 MO used in this study was designed as follows: 5’-GTGCATACACAAATCCAGGAGGGAA.

A morpholino-insensitive Tbx2 construct, MObs-Tbx2, was generated by PCR using KOD Hot Start Polymerase (EMD, Rockland, MA), with the following primers: MO-Tbx2- FP (Phosphorylated) TTTGTGTATGCAACCCGATGAGAGATCCAGCTTTCCCGGGGGG and MO-Tbx2a-RP: TCCAGGAGGGAATGCAAAAAAGAACAAGTAGCTTGTATTC.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

*Xenopus laevis* embryos were staged according to (Nieuwkoop and Faber, 1967) and harvested at appropriate stages according to morphological criteria. RNA was prepared using RNA Bee RNA
isolation reagent (Tel-Test Inc.). RT-PCR was performed as described (Wilson and Hemmati-Brivanlou, 1995). Primer Sequences designed for this study are as follows:

Xmab21l3-F: GGAGGATGAGTAGGATAAAAGTGGTG
Xmab21l3-R: TATGCCGCTCTTCTGATGCCCAG

Xtbx2-F: CCTGGACAGCTGCCTTATTC
Xtbx2-R: CGGCTTCAACTAAGGATGGA

Bix4-F: AGGACCTCCTGTCTTGCCC
Bix4-R: AG ATGCTACAGGCTGGAGCAA

goosecoid-F: 5’-TCTTATTCCAGAGGAACC-3’
goosecoid-R: 5’-AGAGTTCATCTAGAGAG-3’

Xwnt8-F: 5’-GTTCAGCATTAACCCGGAT-3’
Xwnt8-R: 5’-CTCCTCAAATTCATTCTCG-3’

sox2-F: 5’-GAGGATGGACACTTATGCCCAC-3’
sox2-R: 5’-GGACATGCTGTAGGTAGGCGA-3’

sox3-F: 5’-ATCCCATTGACAAGGACCTG-3’
sox3-R: 5’-ATACGAACCAAAGGGGGAAA-3’

odec-F: 5’-AATGGATTTTCAGAGACCA-3’
odec-R: 5’-CCAAGGCTAAAGTTGCAG-3’

chordin-F: 5’-CAGTCAGATGGAGCAGGATC-3’
chordin-R: 5’-AGTCCCATTGCCCGAGTTGC-3’

fgf4-F: 5’-CGGAAGGATAATGGCATGC-3’
fgf4-R: 5’-TTTTGCCCAGAGCGATGTAC-3’

Szl-F: 5’-CATGTCGGAGTCTTCTGC-3’
Szl-R: 5’-GGATGAACGTTGCTCAGGAGCAG-3’

Xbrachyury-F: 5’-GGATCGTTATCACCTCTG-3’
Xbrachyury-R: 5’-GTGTA GTCTGTCGAGCA-3’
sox17β-F: 5’-GTCATGGTAGGAGAGAAG-3’
sox17β-R: 5’-TCTGTGTAGGGCATCATG-3’

VegT F: 5’- CAAGTAAATGTGAGAAACCTG-3’
VegT R: 5’- CAAATACACACACATTTCCCGA-3’

XDelta F: 5’-CTGTCCCCTGGCCTACATT-3’
XDelta R: 5’-CCCTCACACGACAACCAAC-3’

Xenopus epidermal keratin F: 5’- CACCAGAAGACAACGACTAC-3’
Xenopus epidermal keratin R: 5’- CAACCTCCCAGTCAACCAAC-3’

BMP-4 F: 5’-GATCTCAAGTCTAGGCAC-3’
BMP-4-R: 5’-GATCTCAGACTCAACGGCAG-3’

Xvent2: F: 5’TGAGACTTGGCCTACGTCTG-3’
Xvent2: R: 5’CTGTGGTAATGGCTGCT-3’

Wnt11- F: 5’GAAGTCAACCTGCTGAATTCG-3’
Wnt11- R: 5’GCAATGTCAAGTCTGCTG-3’

Derrière-F: 5’-TGCGCTCTGGCTAATGGC-3’
Derrière-R: 5’-CTATGGGCTGCTATGGTTC-3’

Tbx3-F1: 5’CACACAAAAGCCTCCTGTG-3’
Tbx3-R1: 5’ATGGGATTGGTATGGAGG-3’

Tbx3-F2: 5’GTCCAAAGTGTGACCTCCATG-3’
Tbx3-R2: 5’ACTGCAATGAATTCTGCTCA-3’

The primer sequences described above were obtained from the following: *ODC, Xbra, Wnt8, chordin, goosecoid* and *Sox17β* (Suri et al., 2004). *sox2, sox3* and *Sizzled* (Sridharan et al., 2012).

*VegT* (Zhang et al., 1998); *Delta* (Tao et al., 2005); *Epidermal Keratin* (Takahashi et al., 2015); *Bmp4* (Fainsod et al., 1997); *Xvent2* (Miyazaki et al., 2012); *XWnt11* (Afouda and Hoppler, 2011); *Derriere* (Sun et al., 1999). All other sequences were designed by me.
Separation and analysis of outer and inner ectodermal layers:

As stage 10.5, animal caps were cut and placed in CMFM (Ca\(^{2+}\) Mg\(^{2+}\)) free media. This treatment separates the deep layer and the superficial layer of the ectoderm. The separated layers were collected and snap frozen immediately (Sargent et al., 1986). RT-PCR was then performed as described (Wilson and Hemmati-Brivanlou, 1995).

In Vitro translation:

In-vitro transcribed RNA was translated in the presence of \(^{35}\)S, amino acid mix (-LEU), Rnase-Inhibitor and Rabbit reticulocyte mix (L4960-Promega); separation and analysis of products was achieved using SDS-PAGE and autoradiography.

Cycloheximide assays:

Embryos were injected at early cleavage stage with \(tbx2\) RNA (1ng) into the animal pole. Animal cap explants were dissected at mid-blastula stage and treated in cycloheximide (10ug/ml) till early gastrula stages, at which point, they were harvested. Five explants of each sample were collected for the preparation of cDNA prior to being processed by RT-PCR.
Chapter III:
Tbx2 is required for the suppression of mesendoderm during early Xenopus development

3.1 Background
In triploblastic organisms, all tissues, with the exception of the germ cells, derive from the three primary germ layers: ectoderm, mesoderm, and endoderm. Studies in embryos of the frog Xenopus laevis, have been instrumental in our understanding of vertebrate germ layer formation.

Prior to gastrulation, animal pole cells in the Xenopus blastula embryo are specified to become ectoderm, while vegetal pole cells are specified to become endoderm. The mesodermal layer arises from the so-called “marginal zone,” the equatorial cells at the border of the ectoderm- and endoderm-forming regions.

Members of the T-box family of DNA-binding proteins, including Xbrachyury (Xbra) and VegT, have been shown to play a prominent role in the appropriate partitioning of the primary germ layers in the Xenopus embryo; notably, these proteins all function as transactivators (reviewed in Naiche et al., 2005). For example, VegT directly induces the transcription of endodermal genes, as well as genes encoding Nodal-related factors that are secreted and act on the overlying cells of the equatorial region (the marginal zone) to induce mesoderm (Zhang et al., 1998). Nodal-related mesoderm induction, in turn, stimulates expression of another T-box transcription factor, Xbrachyury, in the marginal zone. In mice, where brachyury was first identified, homozygous mutants for brachyury display a complete loss of the posterior mesoderm due to primitive streak
defects, lack a notochord and other mesodermal derivatives, and die in utero (Showell et al; 2004). In *Xenopus*, injection of *Xbrachyury* mRNA results in ectopic ventral mesoderm formation (Cunliffe and Smith, 1992). Injection of RNA encoding either an Engrailed repressor domain-*Xbrachyury* fusion construct, or a *Xbrachyury* mutant truncated at the C-terminus and lacking transactivation activity, leads to the loss of mesodermalizing activity, suggesting that Brachyury plays an essential role in mesoderm formation (Rao, 1994; Conlon et al; 1996); surprisingly, expression of the C-terminal truncation mutant additionally leads to neuralization of animal pole ectoderm (Rao, 1994; Conlon et al; 1996). Another T-box protein, Eomesodermin (Eomes) is also important for mesendoderm development: ectopic expression of Eomes in blastula stage *Xenopus* explants or zebrafish embryos activates transcription of mesodermal and endodermal genes, while inhibition of Eomesodermin function leads to defective mesodermal gene activation (Conlon et al.,2001; Ryan et al.,1996; Bjornson et al., 2005). Notably, chromatin profiling studies in *Xenopus* have demonstrated significant overlap in the genomic binding sites of VegT, Xbrachyury and Eomes, suggesting that these T-box proteins, and perhaps others, activate common target genes during early embryogenesis (Gentsch et al., 2013).

In the early *Xenopus* embryo, active restriction of inappropriate germ layer formation plays a critical role in the differentiation of the ectoderm. Maternal factors including Coco, Ectodermin, SRF, and the zygotic factor XFDL56 repress mesoderm in the early embryo via distinct mechanisms, thus allowing for proper ectodermal development (Bell et al., 2003, Dupont et al., 2005, Yun et al., 2007, Sasai et al., 2008). Studies in our lab and by others have demonstrated
that misexpression of the Fox family DNA-binding protein Xema/Foxi1e stimulates ectodermal
differentiation in cells fated to give rise to mesoderm, while Xema knockdown leads to the
ectopic formation of mesoderm and endoderm in the embryonic ectodermal field (Suri et al.,
2005, Mir et al., 2007). These studies again point to a requirement for suppression of
mesendodermal fate during ectodermal differentiation; as Xema functions as a transcriptional
activator, it likely mediates mesendoderm suppression indirectly, via activation of one or more
transcriptional targets (Suri et al., 2005). A prominent role for transcriptional repression during
ectodermal differentiation has not previously been demonstrated.

We report here the function of the T-box family factor Tbx2 during ectodermal development.

\textit{tbx2} is expressed in the presumptive ectoderm at blastula and gastrula stages. Misexpression of
Tbx2 inhibits differentiation of both ventral ectoderm and growth factor-induced mesendoderm,
and promotes neuralization, the latter via repression of Bone Morphogenetic Protein (BMP)
pathway activity. Tbx2 functions as a transcriptional repressor, and appears to regulate a set of
target genes that overlap with those regulated by transactivating proteins, including Xbrachyury
and VegT. Finally, Tbx2 knockdown leads to ectopic mesendoderm differentiation; taken
together, these studies demonstrate that Tbx2 is sufficient and necessary for the transcriptional
suppression of inappropriate germ layer formation in the presumptive ectoderm.

3.2 Results
3.2.1: Expression of *Xenopus tbx2*

Previous studies demonstrated a requirement for Foxi1e/Xema, functioning as a transcriptional activator, in the suppression of ectopic mesendoderm in *Xenopus laevis* (Suri et al., 2005). We previously performed gene chip-based screens to isolate transcripts that are downregulated following Xema knockdown; isolates from these screens are expected to include potential mediators of Xema function that are expressed in the presumptive gastrula ectoderm (Sridharan et al., 2012). One transcript that was strongly downregulated following Xema knockdown encodes the previously identified T-box protein, *tbx2* (Bollag et al., 1994). We performed animal cap assays on embryos injected with translation-blocking Xema morpholino oligonucleotides and confirmed that *tbx2* is strongly downregulated following Xema knockdown, confirming the results of our microarray analysis (Figure. 3.1A). We note that *xema* overexpression in animal cap explants does not lead to an enrichment of *tbx2* expression (data not shown). These data indicate that Xema is necessary but not sufficient for *tbx2* expression.

Earlier studies have reported that *Xenopus tbx2* expression initiates zygotically, and have detailed the spatial expression of this transcript from neurula through tadpole stage (Takabatake et al., 2000); our studies support these findings (data not shown). While preparing this manuscript, a recent study was published by Cho and colleagues, which reported the expression of *tbx2* in the ventral ectoderm (Cho et al., 2017). Our whole mount in situ hybridization studies demonstrate that *tbx2* is highly expressed throughout the animal pole ectoderm during early gastrula stages, in agreement with expression patterns reported in *X. tropicalis* (Showell et al.,
2006) (Figure 3.1B); *Xbra* expression is restricted to the marginal zone at similar stages, as expected (Figure 3.1B) (Smith et al., 1991). To confirm the exclusion of *tbx2* transcripts from vegetal pole cells, RT-PCR was performed on gastrula-stage animal and vegetal pole explants. *tbx2* expression is observed in animal pole (“cap”) explants and is excluded from the vegetal pole with the latter being the site of VegT expression (Figure 3.1C) (Clements et al., 1999). The ectoderm consists of two populations of cells: an outer, or superficial, polarized layer that forms epithelial cells and deep layer, which forms non-epithelial cells. These two cell types differ in their developmental fate with primary neurons deriving only from the deep cells, and secondary neuronal precursors derived from both the deep and superficial layers; these layers intertwine during neurulation to give rise to a monolayer (Chalmers et al., 2002). To identify the layer or layers in which *tbx2* is expressed, the two layers of the gastrula stage ectoderm were separated and analyzed by RT-PCR; in contrast to *X. tropicalis*, where *tbx2* expression is observed in the outer (epithelial) layer of the animal cap (Showell et al., 2006), *X. laevis* *tbx2* expression is limited to cells in the deep (sensorial) layer (Figure 3.1D). In sum, *tbx2* is expressed zygotically in the region fated to become sensorial ectoderm during *Xenopus* development.

3.2.2: Tbx2 inhibits mesendoderm gene expression

Given that other T-box proteins play a critical role in the patterning of the mesoderm and endoderm, and that Tbx2, to our knowledge, is the first T-box factor with expression throughout the gastrula-stage ectoderm, we endeavored to establish the function of Tbx2 during germ layer
Figure. 3.1. *tbx2* expression in the blastula and gastrula stage ectoderm is dependent on Xema activity. (A) Xema morpholino-mediated knockdown (Xema MO) inhibits expression of *tbx2*. RT-PCR analysis of early gastrula stage explants. Ornithine decarboxylase (ODC) was used as a loading control (Bassez et al., 1990); *Sox17β* is an endodermal marker (Hudson et al., 1997); Chordin is a dorsal endomesodermal marker (Sasai et al., 1994); *Xbra* is a pan mesodermal marker (Smith et al., 1991); *Xmab2113* is a target of Xema (Sridharan et al., 2012; Suri et al., 2005). (B) Whole-mount *in situ* hybridization of early gastrula stage embryos (stage 10.5). *tbx2* expression is seen as a blue stain throughout the animal pole of albino embryos; *tbx2* expression is excluded from the vegetal pole. Expression of the panmesodermal marker *Xbra* is only detected in the marginal zone of gastrula stage embryos, as expected (Smith et al., 1991). (C) RT-PCR analysis of *tbx2* in late blastula stage explants. *tbx2* is expressed in the animal cap and excluded from vegetal pole explants; *vegT* is only expressed in vegetal explants, as expected (Zhang and King, 1996). (D) Inner and outer layers of early gastrula stage ectoderm was separated and analyzed by RT-PCR for expression of *tbx2*. *tbx2* is expressed in the inner layer of the ectoderm; *Delta* is only expressed in the inner layer and serves as a control and *notch* is expressed primarily in the outer layer (Chalmers et al., 2002; Ossipova et al., 2007).
development. Based on the spatial distribution of \textit{tbx2} and microarray analysis demonstrating that Xema is required for early expression of \textit{tbx2}, we hypothesized that Tbx2 might, like Xema, suppress mesendoderm. Incubation of blastula stage animal caps with the TGFβ ligand Activin results in a dose-dependent induction of mesendodermal fate (Ariizumi et al., 1991a, b). Animal caps derived from embryos injected with \textit{tbx2} RNA at early cleavage stages show a marked reduction in levels of the Activin-induced ventral mesodermal markers \textit{Xbra} and \textit{Xwnt-8}, the dorsal mesodermal marker \textit{Chordin} and the endodermal marker \textit{Sox17β} (Figure. 3.2A) (Smith et al., 1991; Christian et al., 1991; Smith and Harland, 1991; Sasai et al., 1994; Hudson et al., 1997). These data demonstrate that \textit{tbx2}, expressed in the animal pole ectoderm, can suppress mesodermal and endodermal fate.

The primary secreted mesendoderm-inducing signal in \textit{Xenopus} is believed to be a member of the Nodal-related branch of the TGFβ superfamily (Whitman, 2001); FGF receptor activation can also induce mesoderm in animal cap explants, and FGF is required in the marginal zone for the differentiation of this germ layer (Harland and Gerhart, 1997). Misexpression of \textit{tbx2} effectively inhibits FGF-mediated mesoderm induction, as well, suggesting that inhibition of mesoderm by Tbx2 is downstream or independent of the Nodal-related and FGF signaling networks (Figure. 3.2B).
**Figure. 3.2. Ectopic Tbx2 suppresses mesendoderm induction.** (A) Tbx2 inhibits Activin-induced mesendoderm. RT-PCR analysis of animal cap explants dissected at late blastula stages and cultured until midgastrula stages. Activin (0.5ng/ml) was added to stage 8 animal caps excised from uninjected embryos or from embryos injected with *tbx2* RNA into the animal pole of both blastomeres at the two-cell stage. (B) Inhibition of FGF-mediated mesoderm induction by Tbx2. RT-PCR analysis of animal cap explants dissected at late blastula stages and cultured until midgastrula stages. bFGF (10ng/ml) was added to stage 8 animal caps excised from uninjected embryos or from embryos injected with *tbx2* RNA, as listed. Unless otherwise noted, 1ng of *tbx2* RNA was used in this and in subsequent experiments.
3.2.3: Tbx2 promotes neural fate

Expression of the epidermal-specific marker, *epidermal keratin* (Jonas et al., 1985), is reduced in animal pole explants derived from tbx2 RNA-injected embryos in the presence or absence of Activin (Figure. 3.2A and data not shown). We therefore questioned the role of Tbx2 in the patterning of the ectoderm itself. It is well established that the ectoderm gives rise to two major, distinct fates: epidermis, ventrally, and neural ectoderm, dorsally (Heasman, 2006). Since Tbx2 suppresses mesoderm, endoderm and ventral ectoderm, we asked if Tbx2 might play a role in promoting the development of neural tissue. To test this possibility, animal caps excised from embryos injected with tbx2 RNA were analyzed for neural markers. We find that Tbx2 induces the pre-neural markers *sox2* and *sox3*, indicating that Tbx2 promotes dorsal ectodermal fate (Figure. 3.3A) (Uwanogho et al.; 1995; Wills et al., 2010).

Previous studies have shown that a critical, initial step in the specification of neural fate is the inhibition of BMP signaling (Weinstein and Hemmati-Brivanlou, 1999). To better understand how Tbx2 promotes neural fate, we first analyzed the effects of Tbx2 on targets of BMP signaling. We find that Tbx2 suppresses markers of BMP4 activity, including *sizzled*, *Xvent-2* and *bmp-4* (Figure. 3.3B) (Marom et al., 1999; Reversade et al., 2005; Rastegar et al., 1999; Rogers et al., 2009). To further explore this repression of BMP signaling, we examined the effects of Tbx2 on the expression of a modified *Xvent-2*-Luciferase promoter lacking Wnt-
responsive TCF/LEF binding sites (TCFm - Vent2-LUC), but responsive to BMP pathway activation (Hikasa et al., 2010). Analysis of whole embryo lysates co-injected with the TCFm - Vent2-LUC construct and tbx2 RNA demonstrate that Tbx2 strongly represses TCFm - Vent2-LUC expression (Figure. 3.3C). Individual T-box proteins bind to distinct T-box binding elements (TBEs) in the promoters of target genes and regulate their expression (Abrahams et al., 2010). Since Tbx2 strongly represses TCFm - Vent2-LUC, we examined the promoter sequence for candidate T-box binding elements (TBEs). Our analysis revealed a predicted TBE site (GGGTGA) which, in another context, is recognized by the Tbx2 protein (Carreira et al., 1998). To test whether Tbx2 can directly repress transcription of Xvent2 through the predicted TBE, this site was mutated (GGGTGA to GGGGTC) in TCFm - Vent2-LUC using a PCR-based mutagenesis strategy (TBEm - Tcfm - Vent2-LUC), thereby likely eliminating the affinity of this site for Tbx2. Embryos were injected with either TCFm - Vent2-LUC or TBEm-TCFm-Vent2-LUC, both in the presence and absence of tbx2 RNA (Figure. 3.3D). Tbx2 retains the ability to repress Luciferase expression in the presence or absence of the putative Tbx2-binding site, suggesting that Tbx2 may regulate Xvent-2 expression indirectly, via intermediary factors.

3.2.4: Tbx2 is required for the suppression of mesendoderm in the animal pole

To determine whether Tbx2 is necessary for ectodermal development, we utilized a morpholino oligonucleotide designed to block translation of tbx2 (Tbx2MO). We first confirmed that Tbx2 MO binds and inhibits translation of tbx2 RNA; as expected, Tbx2MO blocks translation of tbx2 mRNA that contains the Tbx2MO binding site (MObs-Tbx2) in vitro (Figure. 3.4A);
**Figure. 3.3. Tbx2 promotes neural fate.** (A) Ectopic \( t bx2 \) induces the pre-neural markers, \( sox2 \) and \( sox3 \). RT-PCR analysis was performed on animal cap explants from uninjected embryos and from embryos injected with \( t bx2 \) RNA; explants were harvested at gastrula stages. (B) Tbx2 represses the BMP targets \( bmp4 \), \( sizzled \) and \( vent-2 \) (Lee et al., 2002; Collavin and Kirschner, 2003). (A) and (B) show RT-PCR analysis of mid-gastrula stage animal caps. (C). Tbx2 misexpression inhibits expression of a Vent-2 luciferase reporter fusion protein (TCFm-Vent2-LUC); this construct includes a mutation in a TCF binding site that renders it insensitive to Wnt activation (Hikasa et al., 2010). Truncated BMP receptor (tBR) was used as positive control (Graff et al., 1994). Embryos at the 2-cell stage were injected with 5pg of pRLTK, 50 pg of TCF and 1ng \( t bx2 \) RNA or 1ng tBR RNA in the animal pole. (D) TCFm-Vent2-LUC and TCFm-Vent2-LUC with a mutation in the putative T-box binding element (TBEm-TCFm-Vent2-LUC) were injected in the absence or presence of \( t bx2 \) RNA at the 2-cell stage and collected for analysis of Luciferase expression. For (C) and (D), whole embryo lysates were assayed in triplicate for Firefly and Renilla luciferase activity at mid-gastrula stages. Samples of five embryos each were collected in triplicate at stage 11 for analysis; error bars indicate standard error.
consistently, \textit{tbx2} mRNA lacking the morpholino binding site produced a protein of the correct size even in the presence of Tbx2MO (Figure 3.4A). Embryos injected with the Tbx2 morpholino do not develop normally beyond gastrulation, and form none of the hallmark embryonic structures seen at subsequent stages of development (data not shown); we were not, however, able to rescue this phenotype and are thus not able to definitively attribute these defects to the loss or reduction of Tbx2. We next attempted to assay the effects of Tbx2 knockdown in explant assays. We reasoned that if Tbx2 suppresses mesendoderm, loss of Tbx2 might promote the differentiation of ectopic mesendoderm. Consistent with this hypothesis, we find that morpholino-mediated knockdown of Tbx2 leads to elevated expression of mesodermal markers in animal cap explants, including \textit{Xbra}, \textit{Xwnt-8}, \textit{chordin}, and \textit{goosecoid}, and induction of the endodermal marker \textit{sox17β} (Figure 3.4B, lane 2). To confirm that the ectopic mesendoderm is due to the loss of \textit{tbx2}, we performed a rescue experiment: co-injection of \textit{tbx2} RNA inhibits Tbx2MO-mediated mesendodermal gene expression (Figure 3.4B, lane 3). Overall, these results demonstrate that Tbx2 is required for the suppression of inappropriate mesoderm and endoderm in the presumptive ectoderm.

\textbf{3.2.5: Tbx2 functions as a repressor}

We next sought to determine the mechanisms by which Tbx2 regulates gene expression during early development. Tbx2 has been shown to function as a transcriptional repressor in several distinct biological contexts (Carreira et al. 1998; Jacobs et al. 2000; Sinha et al. 2000); however,
Figure 3.4. Tbx2 knockdown leads to ectopic expression of mesendodermal marker genes.

(A) Tbx2 morpholino (Tbx2MO) blocks translation of tbx2 RNA in vitro. Lane 1: translation of tbx2 transcript containing the morpholino binding site; lane 2: translation of tbx2 transcript containing the morpholino binding site in the presence of morpholino; translation of tbx2 transcript lacking the morpholino binding site. The diagram below is a Figure representation of the results in A. (B) Mesendodermal markers are upregulated in ectodermal explants derived from Tbx2 morpholino-injected embryos. RT-PCR analysis of animal cap explants dissected at late blastula stage and harvested until mid-gastrula stage. Embryos were injected with Tbx2 morpholino (80ng) at early cleavage stages. For the rescue experiment, 80ng of Tbx2 morpholino and 1ng of tbx2 RNA were co-injected at early cleavage stages.
structural studies by Paxton and colleagues have identified both activator and repressor domains in Tbx2 (Paxton et al., 2002). In order to determine whether Tbx2 functions as an activator or a repressor in the context of germ layer development, the DNA-binding domain (DBD) of Tbx2 was fused to either the Engrailed (EnR) repressor domain or the VP16 activator domain (Kessler, 1997); RNA transcribed from these constructs was injected into embryos for analysis in the animal cap assay. In Activin-treated caps, tbx2-DBD-EnR expression mimics the repression of mesendodermal genes observed following misexpression of wild-type tbx2, suggesting that Tbx2 functions as a repressor during early embryonic development (Figure. 3.5A). Animal cap explants from embryos injected with tbx2-DBD-VP16 show markedly increased expression of ventral, BMP targets (bmp4 and vent-2), mesoderm, and endodermal genes (Figure. 3.5B), resembling the effects seen following morpholino-mediated knockdown of Tbx2 (Figure. 3.4B). Taken together, these data suggest that Tbx2 functions as a repressor during early development.

3.2.6: Tbx2 represses direct targets of transactivating T-box proteins

Identification of Tbx2 targets constitutes an important step in understanding how Tbx2 exerts its function. The DNA binding domains of Tbx2 proteins are comprised of approximately 180 amino acid residues, and are highly conserved among closely-related T-box family members (Bollag et al., 1994); for example, the DNA binding domains of Tbx4 and Tbx5 share 94% identity (Papaioannou and Goldin, 2008). Consistently, target DNA sequences for closely-related T-box proteins are also highly conserved (Conlon et al., 2001; Abrahams et al., 2010); for
Figure 3.5. Tbx2 functions as a repressor during germ layer differentiation. (A) Expression of Tbx2-DBD-EnR inhibits mesendoderm formation. RT-PCR analysis of animal caps dissected at late blastula stages and cultured until mid-gastrula stages. tbx2-DBD-EnR RNA (1ng) was injected at early cleavage stages, as indicated. Activin (0.5ng/ml) was added to stage 8 animal caps, as indicated. (B) Expression of Tbx2 DBD-VP16 inhibits BMP target gene expression, and stimulates expression of mesodermal and endodermal marker genes. RT-PCR analysis of animal caps dissected at late blastula stages and cultured until mid-gastrula stages. tbx2-DBD-VP16 RNA (1ng) was injected at early cleavage stages, as indicated.
example, Brachyury, VegT and Eomesodermin preferentially bind to the same core motif TCACACCT (Tada et al., 1998; Casey et al., 1999; Conlon and Smith, 1999; Tada and Smith, 2001). Since Brachyury, VegT, and Eomesodermin share similarity in their DNA binding domains and bind to the same core sequence, we hypothesized that Tbx2, with a DNA binding domain that is very similar to that of the T-box transactivators listed above, may bind to an overlapping set of genomic targets (Showell et al., 2004; Abrahams et al., 2010; Tada et al., 1998; Tada and Smith, 2001). In support of this model, we find that ectopic Tbx2 represses Activin-induced induction of Xwnt11, a direct target of Xbrachyury, derriere, a direct target of VegT and bix4, (Brachyury inducible homeobox containing gene), a target of both Brachyury and VegT (Tada and Smith, 2000; White et al., 2002; Zhang and King, 1996; Casey et al., 1999) (Figure. 3.6A). These results suggest that Tbx2, VegT, and Brachyury share at least a subset of target genes, and that Tbx2 represses the expression of these genes, allowing for proper differentiation of the embryonic ectoderm.

3.2.7: Repression of bix4 by Tbx2 is mediated by T-box binding sites on the bix4 promoter

As described above, both Brachyury and VegT bind to regulatory regions of the bix4 gene and induce its expression in mesoderm and endoderm (Tada et al., 1998; Casey et al., 1999). Based on our earlier results, we reasoned that Tbx2 might directly regulate bix4 expression in the cells of the presumptive ectoderm. To address this possibility, we performed Luciferase assays on lysates derived from gastrula stage embryos that had been injected at early cleavage stages with a bix4-Luciferase reporter fusion construct (bix4-LUC) in the presence or absence of tbx2 RNA
As expected, ectopic Tbx2 strongly represses expression of bix4-luciferase (Figure 3.6B). The Bix4 upstream regulatory region contains three T-box binding elements located within 200 bp of the transcription start site, labeled previously as Td, Tm and Tp (T-box distal, medial, and proximal, respectively) (Casey et al., 1999). Mutational studies of the three sites suggested that the most distal site (Td) likely plays a role in restricting expression of Bix4 (Casey et al., 1999). We speculated that Tbx2 might bind to the distal site (Td) of bix4 and repress its expression. To test this hypothesis, we injected embryos with the Td-mutant construct in the presence or absence of tbx2 RNA, and performed Luciferase assays on lysates derived at gastrula stages. In agreement with Casey et al., (1999), we find that the Td construct alone has higher basal levels of activity than does the wild-type construct (Figure 3.6C). tbx2 RNA inhibits expression of wild-type bix4; expression of Td-bix4 is reduced, but still prominent, upon co-injection of tbx2 RNA, suggesting that Tbx2 represses bix4 expression in part via the Td T-box binding site. We next generated a construct in which all three known T-Box binding sites, distal, middle and proximal, were mutated (TdTmTp-bix4). Injected alone, this construct, like Td-bix4, has higher basal levels of activity than does wild-type bix4 (Figure 3.6C). TdTmTp-bix4 Luciferase activity, however, is completely insensitive to Tbx2-mediated repression; this suggests that Tbx2 inhibits bix4 expression via multiple T-box binding sites on the bix4 promoter.
Figure. 3.6. *Tbx2* represses downstream targets of *VegT* and *Brachyury*. (A) *Tbx2* represses expression of *derriere*, *Xwnt11*, and *Bix4* (Casey et al., 1999; Zhang and King, 1996). RT-PCR analysis of animal caps dissected at late blastula stages and cultured until mid-gastrula stages, as indicated. Activin (0.5ng/ml) was added to stage 8 animal caps, as indicated. (B) *Tbx2* represses expression of a *Bix4* reporter construct. Embryos at the 2-cell stage were injected with *bix4*-LUC (20pg) and pRLTK (7pg), in the absence and presence of *tbx2* RNA (250 pg). Whole embryo lysates from gastrula stage embryos were assayed in triplicate for Firefly and Renilla luciferase activity. (C) Mutation of three T-box binding sites renders *bix4* insensitive to repression by *Tbx2*. Embryos were injected at the 2-cell stage with pRLTK (7pg) and *bix4*-LUC (20pg), *Td*-LUC (20pg), or *Tdmp*-LUC (20pg) in the absence and presence of *tbx2* RNA. Whole embryo lysates from gastrula stage embryos were assayed in triplicate for Firefly and Renilla luciferase activity. *Tbx2* blocks the expression of *Td*-LUC less efficiently than it does *bix4*-LUC, while mutation of the three T-box sites renders *Tdmp*-LUC insensitive to repression by *Tbx2*. Samples of five embryos each were collected in triplicate at stage 11 for analysis; error bars indicate standard error.
3.3 Discussion and future directions

We report here the localization and activity of the T-box DNA binding factor Tbx2 during germ layer differentiation. *tbx2* is expressed in the deep layer of the presumptive ectoderm in *Xenopus laevis* embryos. Tbx2 knockdown results in the ectopic expression of mesodermal and endodermal marker genes, while Tbx2 misexpression inhibits epidermal fate, promotes neuralization, and suppresses both Activin-induced mesendoderm induction and FGF-induced mesoderm induction; Tbx2 therefore functions as a transcriptional repressor during mesendoderm suppression. Taken together, our studies demonstrate that appropriate germ layer differentiation in the *Xenopus* embryo is dependent upon Tbx2-mediated repression of extra-ectodermal fate.

Endoderm and mesoderm differentiation are regulated by multiple transactivating T-box proteins, including VegT, Xbrachyury, and Eomesodermin, all of which share DNA target sequences similar to that of Tbx2 (Gentsch et al., 2013; Ryan et al., 1996). These T-box factors show restricted expression, with *tbx2* expressed in the presumptive ectoderm, and *Xbrachyury*, *vegT* and *eomesodermin* expressed in the presumptive endoderm and/or mesoderm (Showell et al., 2004). We report here that repression of *bix4*, a previously described target of both Brachyury and VegT, by Tbx2 is mediated via multiple T-box binding sites in the *bix4* promoter (Tada et al., 1998; Casey et al., 1999). It is well established that transactivating T-box proteins bind to and induce expression of mesendodermal target genes, including *bix4* (Tada and Smith, 2001); our studies build on and refine this model of T-box function, and suggest that repressor T-box
proteins including Tbx2 suppress expression of these same genes, and thus inhibit mesendodermal differentiation, in the cells of the presumptive ectoderm (Figure 3.7).

Direct repression of mesendodermal target genes may not be the only mechanism by which Tbx2 inhibits inappropriate germ layer differentiation. During preparation of this manuscript, Cho and colleagues reported that Tbx2 limits FGF-mediated neural caudalization via transcriptional suppression of the flrt3 gene (Cho et al., 2017). flrt3 does not appear to be expressed in the presumptive ectoderm at gastrula stages (Böttcher et al., 2004); nevertheless, this finding raises the possibility that Tbx2 inhibits mesendoderm both through direct binding to mesendoderm-specific target genes and through indirect regulation of FGF signaling, the latter shown previously to be required for mesodermal differentiation (Heasman, 2006).

Suppression of ectodermal BMP activity by Tbx2 may be mediated through direct repression of BMP target genes. The absence of de-repression following mutation of the sole candidate TBE site within the region of the vent-2 promoter shown to be sufficient for regulation by BMP (Lee et al., 2002; Henningfeld et al., 2000), however, suggests that an alternate mechanism may underlie BMP signal inhibition by Tbx2. Studies have demonstrated that the T-box proteins Brachyury and Eomesodermin interact with the BMP signal transducer Smad1 during mesodermal and endodermal differentiation, respectively (Messenger et al., 2005; Faial et al., 2015). Another T-box protein, Tbx20, has been shown to inhibit BMP signaling by directly binding to Smad1 and Smad5, sequestering them from association with the co-factor Smad4; this
Figure 3.7. A model of Tbx2-mediated ectodermal specification. Tbx2 binds to and represses target genes in animal pole progenitor cells, thereby inhibiting mesoderm and endoderm formation. In the marginal zone and vegetal pole, other T-box proteins bind to and activate an overlapping set of target genes, stimulating mesodermal and endodermal differentiation.
interaction is mediated by the Tbx20 T-box domain (Singh et al., 2009; Henningfeld et al., 2000). These studies raise the possibility that Tbx2 may also physically associate with Smad1 or other intracellular mediators of BMP receptor activity to abrogate BMP signaling.

Although we have established a requirement for Tbx2 in the suppression of ectopic mesendoderm, the physiological significance of BMP target gene repression in this process is not clear. Tbx2-mediated BMP inhibition may contribute primarily to one of several previously described mechanisms that limit BMP activity in, and thus limit the ventralizing of, the early embryo prior to the secretion of extracellular antagonists from the Organizer (Zhu et al., 1999; Bell et al., 2003, Onichtchouk et al., 1999, Dupont et al., 2005, Sridharan et al., 2012, Heasman, 2006). As epidermal induction occurs in regions of the embryo that express native tbx2, however, it is at present difficult to conclude that Tbx2-mediated BMP suppression plays a central role in neuralization of the dorsal ectoderm.
Chapter IV: Characterizing mediators, regulators and identifying functional domains of Tbx2

4.1 Background
In order to understand early development, it is crucial to understand the molecular mechanisms that pattern the embryo during early embryogenesis. As mentioned in the introduction, signals originating from the underlying endoderm induce mesoderm in the adjacent marginal zone of Xenopus laevis embryos (Slack, 1991). Several members of the TGF-β growth factor superfamily, including Activin, Vg1 and the Nodal-related proteins are sufficient and, in the case of the Nodal-related factors, appear necessary for mesoderm induction (Harland and Gerhart, 1997; Heasman, 2006; Slack, 1991). Work from our lab and by others have shown that inhibition of these and other signals are required for the formation of the ectoderm and therefore appropriate germ-layer specification. This requirement can be observed upon loss of ectodermal specification factors, such as Ectodermin and Xema, which leads to the expansion of mesoderm at the expense of ectoderm (Dupont et al., 2005; Suri et al., 2005). In this thesis, we have added Tbx2, to the list of factors crucial for ectodermal specification; Tbx2 functions, as we have shown, to repress mesendodermal gene expression in the presumptive ectoderm. In Chapter III we identified Bix4, a target of the T-box proteins VegT and Brachyury, as a gene that can be repressed by Tbx2, and a potential in vivo target of repressor T-box proteins. Here, we attempt to address several remaining, important issues regarding Tbx2 structure, function, and regulation:

what transcriptional targets of Tbx2 are central to its role in mesendodermal suppression? What
are the signaling and gene regulatory networks that govern \( tbx2 \) expression? What are the domains of Tbx2 that are important for its function during mesendodermal suppression?

In this Chapter, we identify \textit{brachyury} and \textit{goosecoid} as potential direct targets of Tbx2 in the \textit{Xenopus} embryo in a cycloheximide assay. We then confirm the repression of \textit{goosecoid} in a Luciferase assay using different regions of the promoter (-1500, -492 and -262 bp). Surprisingly, mutation of several previously identified VegT-binding sites (TM123) on -492bp \textit{gsc/luc} (Sudou et al., 2012) did not affect the repressive ability of Tbx2, suggesting that the repression of \textit{goosecoid} may be mediated via unidentified T-box sites. In an effort to identify upstream regulators of Tbx2, we found that loss of BMP signaling leads to loss of \( tbx2 \) expression, suggesting that BMP signaling might be an upstream regulator of \( tbx2 \) expression. Finally, we also describe the construction and characterization of Tbx2 deletion constructs that we utilize to identify the domains responsible for Tbx2 function during germ layer differentiation.
4.2 Results

4.2.1: Tbx2 represses cycloheximide-induced genes

Cycloheximide (CHX) is an inhibitor of eukaryotic protein synthesis; while the precise mechanism of action is not yet fully understood, it has been shown to inhibit translation elongation through binding to the E-site of the 60S ribosomal unit and interfere in translocation of deacetylated tRNA (Klinge et al., 2011; Obrig et al., 1971; Pestova and Hellen, 2003; Schneider-Poetsch et al., 2010). We chose to use cycloheximide to identify direct targets that are likely to be regulated directly by Tbx2. Transcription factors that are already present within the cell are able to regulate the expression of their target genes; however, in the presence of cycloheximide, the target genes cannot be translated, and so cannot activate indirect targets. Thus, genes that are upregulated by transactivators in the presence of cycloheximide are likely to be direct targets. Interestingly, cycloheximide treatment alone is able to increase transcription of the pan-mesodermal marker brachyury and the dorsal mesodermal marker goosecoid (Dawid et al., 1993; Engleka et al., 2001; Tadano et al., 1993), both of which are repressed by Tbx2. We reasoned that if brachyury and goosecoid are direct targets of the transcriptional repressor Tbx2, we should expect to see a decreased expression of these genes. In line with our thinking, we found that in animal caps injected with tbx2 and treated with cycloheximide, there is a decrease in cycloheximide-induced goosecoid and brachyury, suggesting that these genes along with bix4 may be direct targets of Tbx2 (Figure. 4.1).
Figure 4.1. Tbx2 represses the cycloheximide-induced mesodermal genes *goosecoid* and *brachyury*. RT-PCR analysis of animal cap explants injected with *tbx2* during early cleavage stages, excised at late blastula stages, and cultured until midgastrula stages in both the presence and absence of cycloheximide (10 μg/ml). *ODC* was used as loading control. Cycloheximide treatment alone is able to induce the transcription of the panmesodermal gene *brachyury* and the dorsal mesodermal gene *goosecoid* (Dawid et al., 1993; Engleka et al., 2001; Tadano et al., 1993).
4.2.2: Tbx2 strongly represses goosecoid –LUC

The studies above suggest that Tbx2 might directly suppress goosecoid expression in the cells of the presumptive ectoderm. To address this possibility, we performed Luciferase assays on animal cap explants from embryos that were injected at early cleavage stages with a -1.5KB promoter (relative to the transcription start site) containing goosecoid-Luciferase reporter fusion construct (gsc/luc) in the presence or absence of tbx RNA (Sudou et al., 2012; Watabe et al., 1995). The animal caps were then dissected at early blastula and harvested till early gastrula stage. As expected, there was little induction of goosecoid in the absence of Activin (Figure. 4.2A). In agreement with previous studies, the addition of Activin, strongly induced goosecoid expression (Watabe et al., 1995). Goosecoid induction is strongly repressed in the presence of ectopic Tbx2, suggesting that Tbx2 may repress goosecoid expression by directly binding to the promoter. We wanted to find the smallest region in goosecoid promoter that can respond to Activin but still be repressed by tbx2 RNA. For this purpose, we analyzed the -226-nucleotide region of the goosecoid promoter (relative to the transcription start site), the smallest region of goosecoid promoter that is reported to be responsive to Activin (Watabe et al., 1995). In agreement with the published results, we did find that the -226 gsc/luc is able to respond to Activin, but its induction is much lower compared to induction observed by -1500 gsc/luc; we also found that Tbx2 is not able to effectively repress the induction as strongly as it did with -1500 gsc/luc (Figure. 4.2B). In the study published by Watabe et al.,1995, an intermediate construct, -492 gsc/luc is also induced by Activin. Since we wanted to find the region of goosecoid promoter that is responsive to activin and still repressed by Tbx2, we next tested the intermediate construct. As expected, we
did find that the -492 gsc/luc was induced in the presence of Activin (Sudou et al., 2012; Watabe et al., 1995). In the presence of tbx2 RNA, there is a strong repression of Activin-induced expression of gsc/luc (Figure. 4.2C). In a previous study, Sudou et al., 2012 identified three conserved consensus T-box sites, T1, T2 and T3, in -492 gsc/luc, which strongly responded to the T-box activator protein, VegT in a dose dependent manner. A construct in which these sites were mutated (TM123) showed a great reduction in reporter response (Sudou et al., 2012). We wanted to determine whether Tbx2, like VegT, might also bind to the same sites and repress the expression of goosecoid. For this reason, we injected embryos with the TM123 in the presence or absence of tbx2 RNA. Our results indicate that Tbx2 is still able to repress the mutated version of goosecoid, suggesting that Tbx2 mediates its repression by binding elsewhere on the goosecoid construct or via an indirect mechanism. Upon analysis of the promoter upstream of -492 nt, we note that there are three other potential T-box sites in the promoter. We also note that the TM123 construct shows a very high response in the presence of Activin, suggesting that another protein in the T-box family may bind and repress goosecoid expression at the T1, T2, and/or T3 sites.

4.2.3: BMP signaling may regulate the expression of tbx2

tbx2 was initially identified in a microarray screen as a gene whose expression was lost in animal caps injected with morpholino designed to block translation of the transcriptional activator, Xema (Sridharan et al., 2012; Suri et al., 2005). We confirmed this result in animal
A

Relative luciferase activity

B
Figure 4.2. Tbx2 represses gsc-luciferase. Embryos at the 2-cell stage were injected construct containing -1500 promoter of goosecoid fused to luciferase (gsc-Luc) (20 pg). Animal caps were then dissected from blastula stage embryos, and placed in the presence or absence of Activin (20ng/ul) for three hours. Lysates from gastrula stage caps were assayed in triplicate for Firefly and Renilla luciferase. Ten animal caps were used for each trial, so a total of 30 animal caps per condition were analyzed. (B) Tbx2 is less sensitive in repressing -226 gsc/luc compared to -1500 gsc/luc (Compare bars 3 and 7). (C) Tbx2 is able to repress both the intermediate -492 gsc/luc

1 Legend for this picture (Figure 4.2) is on the next page (51).
construct and also -492 gsc/luc construct mutated on all three established T-box sites located within this region (TM123). For all experiments, Prlk (7pg) was used as an internal control and 250pg tbx2 RNA was injected. The luciferase assays in these experiments were repeated in 5 different experiments and were found to have the same pattern shown in this figure. Samples of fifteen caps each were collected in triplicate at stage 11 for analysis; error bars indicate standard error. Cap assays (Figure. 3.1A); however, overexpression of Xema did not lead to increased expression of tbx2 in animal caps (data not shown). This led us to question what signal(s) or transcription factor(s) activate tbx2 expression in the blastula stage embryo. Other studies have suggested that BMP signaling regulates the expression of tbx2; in one study, BMP4-soaked beads implanted into the central mesenchyme of forelimb buds in mice led to an increase in expression of tbx2 (Farin et al., 2013). BMP signaling has also been shown to induce tbx2 expression in pronephric nephrons (Cho et al., 2011). In Ascidians, Tbx2 is expressed in the ventral ectoderm of the tail under the influence of BMP expression and is lost in the presence of the BMP antagonist, Dorsomorphin (Waki et al., 2015). To test the possibility that BMP signaling regulates tbx2 expression in the early Xenopus embryo, we assayed for tbx2 gene expression in animal caps injected with RNA encoding a truncated BMP mutant receptor (tBr), lacking the serine/threonine kinase domain, known to function as a dominant negative receptor (Suzuki et al., 1994). We
found that loss of BMP signaling leads to a loss of tbx2 expression in caps harvested at gastrula stages, suggesting that BMP signaling might indeed be an upstream regulator of tbx2 expression in this context (Figure. 4.3A). As an indicator of tBr activity, we observed an increase in the expression of the neural markers, sox2 and sox3 (Figure. 4.3A); these genes are known to be induced upon loss of BMP signaling (Delaune et al., 2005). To further address whether BMP might function as an upstream regulator of tbx2, we scanned the promoter region upstream region of the tbx2 coding sequence (1170 bp) for potential Smad binding elements (SBE), known to mediate BMP signaling. We found multiple SBE sites - highlighted in pink - (CAGA, GTCT, GCCG, GCCGA, ATGC) that have been shown to bind to Smads (Shirai et al., 2009) (Figure. 4.3B). Interestingly, we also found many potential T-box sites – highlighted in yellow – in the tbx2 promoter sequence, suggesting that T-box proteins, including Tbx2 itself, can regulate tbx2 expression (Figure. 4.3B) (Carreira et al., 1998; Wilson and Conlon, 2002). These results point to the possibility that both BMP-mediated Smad and T-box proteins regulate the expression of tbx2.

**4.2.4: Identifying functional domains of Tbx2**

In contrast with Brachyury and VegT, which are transcriptional activators, Tbx2 is a potent repressor based on our results and others (Carreira et al., 1998). However, the mechanism by which Tbx2 mediates its repression of mesendodermal target genes is still unknown. Based on earlier studies of Tbx2 function, repression may include involvement of the Groucho/TLE family of co-repressors which interact with DNA binding proteins to repress target genes (Fisher and Caudy, 1998); alternatively, Tbx2 may function in this context via histone deacetylation, which
involves modification of the histone – DNA interaction and chromatin rearrangement resulting in decreased availability of the DNA transcription factors (Ayer, 1999). In order to identify the mechanisms underlying Tbx2 function during germ layer differentiation, the domains of Tbx2, specifically the repressor domains, required for this activity must be mapped. Previous studies have identified repressor domains in the C-terminus of Tbx2 (Sinha et al., 2000); however, in another study, an additional repressor domain was identified in the N-terminus (Paxton et al., 2002; Sinha et al., 2000). This led us to examine which domains of Tbx2 are actually important for repressing mesendodermal and BMP target gene expression in the ectoderm. To study this,

Figure. 4.3. Loss of BMP signaling leads to loss of Tbx2 expression. RT-PCR analysis was performed on animal cap explants from gastrula stage embryos injected with tBR (truncated BMP receptor) during early cleavage stages. Loss of BMP signaling leads to an increase in the neural markers, sox2 and sox3 relative to uninjected caps; the levels of bmp4 remained the same and there is a loss of tbx2 expression in tBR injected caps. (B) Smads and T-box proteins could regulate the expression of Tbx2. Shown is the -1170 bp upstream regulatory region of tbx2, with
the starting ATG codon highlighted in red. Potential Smad binding elements are shown in pink; possible binding sites for T-box proteins, including Tbx2, are shown in yellow.

we created deletion constructs as shown in Figure. 4.4.A. RNA from the deletion constructs of Tbx2ΔC or Tbx2ΔN was injected into embryos and analyzed in the absence of Activin for ventral suppression. We find that the loss of the C-terminal domain did affect the repressor activity of Tbx2: BMP4, sizzled and vent-2 which are repressed in the presence of full length Tbx2, maintain high levels of expression in the presence of perhaps due to loss of the C-terminal repressor domain. The loss of the N-terminal repressor domain also had an effect on repression, as we continue to observe high levels of expression of BMP4, sizzled and Vent-2 following expression of N or C terminal deletion constructs (Figure. 4.4 B). We are in the process of addressing how the other deletion constructs generated will play a role in the function of Tbx2. Thus far, our results suggest that both the N and C repressor domains are important for the repressive activity of Tbx2.
Figure 4.4. Tbx2 deletion constructs identify distinct functional domains. (A) The light gray boxes represent the N–terminal repressor and C-terminal repressor domains as defined previously by (Paxton et al., 2002; Sinha et al., 2000). The cross-hatched domains represent areas of unidentified function in Tbx2. The black domain denotes the T-box DNA binding
domain, which is able to bind to promoter region of target genes. (B) RT-PCR analysis was performed on animal cap explants from gastrula stage embryos injected with RNA from full length Tbx2, Tbx2∆C or Tbx2∆N during early cleavage stages. Loss of N or C terminal repressor domain is unable to repress bmp4, sizzled or vent-2.

4.3 Discussion and future directions

In this chapter, we describe the identification of two additional potential targets of Tbx2, brachyury and goosecoid, using a cycloheximide assay. We obtained different truncated versions of goosecoid that have been shown to be active in the presence of Activin (-1500, -492, -226 bp) (Sudou et al., 2012; Watabe et al., 1995). In accordance with data from Watabe et al., 1995, we found that all three constructs show a strong response to Activin; the addition of tbx2 RNA repressed Activin-induced goosecoid expression from all of the constructs, suggesting that Tbx2 is a strong inhibitor of goosecoid. However, a goosecoid construct mutated in all identified T-box sites (TM123), did not have an effect on the repressive capability of Tbx2 (Sudou et al., 2012), suggesting that Tbx2 may bind to other T-box sites on goosecoid or that it acts indirectly to repress promoter activity. Our data also indicate that BMP signaling might be an upstream regulator of tbx2 expression and that other T-box proteins, including Tbx2, may also regulate tbx2 expression. Finally, in this chapter we also describe the analysis of Tbx2 deletion constructs to identify the regions of Tbx2 essential for its function.

It was interesting to find that Tbx2 was still able to repress goosecoid even when the T-box sites on the goosecoid promoter were mutated. These sites were initially shown to be responsive to another T-box protein, VegT (Sudou et al., 2012): VegT was shown to induce the expression of goosecoid, while mutation in the T-box sites (TM123) rendered VegT inactive in inducing
**goosecoid.** Our results could be due to the presence of an uncharacterized TBE that may be present in the promoter that enables the binding of Tbx2 to different sites other than the ones mutated in the -492 gsc/luc construct; alternatively, Tbx2 may repress goosecoid indirectly by interacting with other endogenous DNA-binding proteins or cycloheximide blocks other repressors, and Tbx2 can compensate for the loss of the other protein. It is unlikely that Tbx2 represses goosecoid indirectly, as the cycloheximide assay is designed to reveal direct targets; this points to binding of Tbx2 to a different site on goosecoid as a more likely possibility.

In chicks, application of BMP2 selectively induced Tbx2 and Tbx3 expression in non-cardiogenic embryonic tissue, while the Bmp antagonist Noggin down regulated tbx2 gene activity (Yamada et al., 2000). In mice, the expression of mTbx2 was blocked in bmp2 null mouse embryos; to a lesser extent, Tbx3 gene activity appears to be directed by BMPs during early cardiogenesis. All these studies suggest that BMP could be an upstream regulator of Tbx2. Consistently, we observe a loss of tbx2 expression upon loss of BMP signal. In addition, the identification of several Smad sites on the tbx2 promoter implicate Smads in activating Tbx2 expression. To test whether Smads are important in regulating tbx2 expression, we are currently in the process of building a Luciferase reporter driven by 1.2kb of sequence upstream of the tbx2 transcription start site. We intend to test the responsiveness of this construct to exogenous BMP4 protein, Smads 1,5, 8, and misexpression of T-box proteins, including Tbx2. This study will help us better understand the regulation of Tbx2, a critical factor in early germ layer patterning.
Chapter V: Tbx3 may play a role in specification of ectoderm

5.1 Background

A significant event during embryogenesis is a process called gastrulation during which the three germ layers - ectoderm, mesoderm and the endoderm are established. As described above, T-box family of proteins play a crucial role in patterning the germ layers. *brachyury*, the first T-box gene to be molecularly characterized, is required for the formation of mesoderm and its derived tissues, including the notochord and somites. Mice homozygous for mutations in *brachyury* die shortly after gastrulation and display severe mesodermal abnormalities, including loss of the posterior mesoderm. Another T-box family member, Veg-T, is responsible for proper endoderm formation. Loss of Veg-T results in shortened body axis, extensive defects in head development and a marked reduction in expression of all endodermal genes. In chapter III, we identified another T-box transcription factor, Tbx2, as an important player in the specification of ectoderm. *tbx2* was identified in a microarray analysis as a target of Xema (Suri et al., 2005) (Chapter III).

In addition to *tbx2*, our microarray studies indicated that *tbx3* expression was also downregulated in Xema morpholino-injected embryos. Tbx3 and Tbx2 are closely related members of the Tbx2 subfamily, whose other members include Tbx4 and Tbx5 (Agulnik et al., 1996; Carreira et al., 1998; He et al., 1999). Tbx3, like Tbx2 is defined by the 200-amino acid T-box domain that has
been shown to exhibit DNA binding; both proteins have been shown to function as repressors in other contexts (Pflugfelder et al., 1992; Carreira et al., 1998). Tbx3 has been shown to be essential in development and is an important player in oncogenesis: it is overexpressed in several cancers, contributes to tumor formation and can drive proliferation of cancer cells (Willmer et al., 2016). In humans, having one functional copy of TBX3 is believed to cause an autosomal dominant condition called Ulnar mammary syndrome (UMS), which is defined by defects of the upper extremities that may include hypoplastic or missing ulna (Loyal and Laub, 2014) and is associated with defects in heart, limbs, and mammary gland (Packham and Brook, 2003). In the development of the mouse, *Tbx3* expression begins in the inner cell mass of the blastocyst, and then appears in the extraembryonic mesoderm during gastrulation. Tbx3 plays key roles in both the establishment and maintenance of pluripotency in embryonic stem cells and induced pluripotent cells (Washkowitz et al., 2012) and can inhibit differentiation of progenitor cells, (Davenport et al., 2003; Ivanova et al., 2006; Lu et al., 2011). In *Xenopus laevis*, Tbx3 was originally identified as ET (eye T-box), a necessary component in the transcription factor network regulating eye field development (Li et al., 1997), misexpression of which can induce ectopic and functional eyes (Motahari et al., 2016). While *Tbx3* has been implicated in a variety of developmental contexts, the mechanisms by which it functions during development remains unclear.

In the work described below, we provide evidence to suggest that *tbx3*, expressed in the presumptive ectoderm, functions primarily to repress inappropriate expression of ventral mesodermal and endodermal genes.
5.2 Results

5.2.1: Xema is necessary but not sufficient for the expression of tbx3

tbx3 was identified in our microarray screen as a transcript dependent on Xema activity; this finding was confirmed in animal cap assays (Figure. 5.1A). As a control for Xema morpholino activity, we note the decrease in expression of Xmab21l3, whose expression was previously shown to dependent on the presence of Xema (Sridharan et al., 2012). Two different primer sets were used for tbx3b, both of which show a decrease in tbx3 expression in animal caps upon loss of Xema.

We next examined whether misexpression of Xema leads to upregulation of tbx3 expression. Embryos injected with xema RNA were analyzed for tbx3 expression. We found that Xema misexpression did not lead to an increase of tbx3 expression with either of the primer sets used. Xtox (Xmab21l3) and Pitx1, as expected, do show an increased expression in xema RNA injected caps (Figure. 5.1B) (Sridharan et al., 2012, unpublished lab result). This suggests that Xema is necessary but not sufficient for the expression of the T-box gene, tbx3.

5.2.2: Spatio-Temporal expression of tbx3

To begin to address the role of Tbx3 during germ layer differentiation, it is important to identify whether tbx3 is expressed during early development. Previous studies have shown that tbx3 is expressed zygotically from stage 10 onward in the embryo (Takabatake et al., 2000). To confirm
Figure. 5.1. *tbx3* expression is lost upon Xema knockdown but is not induced by Xema. (A) RT-PCR analysis of animal caps from embryos injected with Xema morpholino. Xmab21l3, the expression of which was previously shown to be dependent upon Xema, is reduced following injection of the Xema morpholino (Sridharan et al., 2012). The two primer sets used to observe Tbx3 expression are both repressed upon loss of Xema. (B). RT-PCR analysis of *xema* RNA-injected animal caps. Xmab21l3 and Pitx1 are both induced by Xema misexpression and serve as positive controls (Sridharan et al., 2012). Neither *tbx2* nor *tbx3* are induced by misexpression of Xema. Embryos were injected at the 2-cell stage and caps were cut at blastula stages and harvested at late gastrula stages.
this finding, we performed RT-PCR analysis from whole embryo lysates to identify the stage at which \textit{tbx3} is expressed. Our studies show that \textit{tbx3} is expressed maternally and significantly increases from stage 10.5 (Figure 5.2 A); by comparison, the exclusively zygotic expression of \textit{tbx2} can be observed from stage 8 onward. We next performed \textit{in situ} analysis of \textit{tbx3} to identify where \textit{tbx3} is expressed in the embryo. \textit{In situ} analysis of gastrula stage embryos revealed expression of \textit{tbx3} in the animal pole, while the vegetal pole is not stained; this staining is very similar to that observed for \textit{tbx2} at this stage (Figure 5.2 B; \textit{tbx2} and \textit{brachyury} staining was shown earlier in chapter III, Figure 3.1B, and is reused in this Figure for the purpose of comparison to \textit{tbx3} expression). \textit{brachyury} expression (shown earlier in Figure 3.1B), as expected, is expressed in the marginal zone (Figure 5.2 B). These results indicate that \textit{tbx3}, like \textit{tbx2}, is expressed in the ectoderm during gastrula stages of development.

\textbf{5.2.3: Activity of \textit{Xenopus laevis tbx3}}

We have previously demonstrated that ectodermally-expressed Xema regulates germ layer formation and patterning (Suri et al., 2005). Our own results, described in this thesis, suggest that Tbx2, localized in the ectoderm, is also able to regulate germ layer formation. We wanted to address the potential function of Tbx3 in germ layer formation; therefore, we misexpressed \textit{tbx3} RNA into the animal pole of early stage embryos and assayed its activity in ectodermal explants, in the presence of the TFGß ligand, Activin. Injection of RNA encoding \textit{tbx3} results in a slight
Figure 5.2. *tbx3* is expressed in the animal cap during blastula stages. (A) RT-PCR analysis of whole embryo lysates from embryos harvested from blastula, gastrula and neurula stages. *tbx2* is expressed zygotically from stage 8 onward, while *tbx3* is expressed both maternally and zygotically. (B) Whole-mount in situ hybridization of gastrula stage embryos showing *tbx3* expression in the animal pole as a blue stain, and its exclusion from the vegetal pole. *tbx2* is also expressed in the animal pole and *brachyury* is expressed in the marginal zone, as published.
increase in the levels of dorsal mesodermal markers by Activin, as demonstrated by the induction of chordin and goosecoid (Figure. 5.3). However, the induction of ventral mesodermal markers, Xbrachyury and Xwnt8 by Activin are reduced in the presence of exogenous Tbx3, and endodermal markers, sox17β and Xhex are not induced in these cultures. Tbx3 slightly induces the expression of ectodermally expressed epidermal keratin, suggesting it may play a role in ventralizing ectoderm. Taken together, these data suggest a role for Tbx3 is dorsoventral patterning of the early Xenopus embryo.

Since Tbx3 gain-of-function enhances dorsal mesoderm formation, we hypothesized that loss of Tbx3 would lead to a reduction in dorsal differentiation. To test for this possibility, we injected Tbx3 morpholino designed to block translation of tbx3 into early stage embryos to study the requirement for Tbx3. Unfortunately, the morpholino used was ineffective in blocking the translation of tbx3 (data not shown). In order to better study whether endoderm and ventral mesoderm suppression and/or epidermal induction by Tbx3 is required for normal development, we will need to obtain different morpholinos, targeting a different site on tbx3, that effectively knock down Tbx3 activity.
Figure 5.3. Ectopic tbx3 represses mesoderm and endoderm induction. RT-PCR analysis was performed on gastrula stage caps injected with tbx3 RNA (1ng) during early cleavage stages. Embryos were allowed to develop till mid-blastula stage, at which point animal caps were dissected and cultured in Activin (0.5ng/ml) until harvest at midgastrula stages.
5.3 Discussion and future directions

In this chapter, we report that the expression of a second T-box gene, tbx3, is also lost following Xema knockdown; moreover tbx3, like tbx2 is also expressed in gastrula stage ectoderm. Misexpression of tbx3 RNA in Activin-treated animal caps represses ventral mesodermal and endodermal genes but, unlike Tbx2, exogenous Tbx3 enhances Activin-induced dorsal mesodermal marker expression. These results point to a role of T-box repressor proteins as crucial players, with both overlapping and non-overlapping functions in the specification of ectoderm.

While the data here suggest a role for Tbx3 in ectodermal specification, the requirement of Tbx3 in this context remains to be addressed. To this end, we plan to inject morpholinos targeting distinct regions of tbx3 and assay for mesendodermal gene induction in the ectoderm. The morpholino used was inactive; however, it remains a possibility that there is some redundancy for T-box protein function in the ectoderm. In other words, endogenous Tbx2 may be sufficient to repress some mesendodermal genes upon injection of Tbx3 morpholino, and this result would not be surprising, as they have been shown to play redundant roles in the formation of heart chambers in mice (Singh et al., 2012). Based on our studies with exogenous tbx3, it is also possible that we will see ectopic expression of only a subset of non-ectodermal markers expressed following successful knockdown of Tbx3; e.g. we may observe an increase of ventrolateral mesodermal markers only. Regardless, further studies need to be performed before we can definitely ascribe a role for Tbx3 in early embryonic patterning, in vivo.
It is interesting to note that *tbx3*, like *tbx2*, is not induced by Xema misexpression. Our data indicate that BMP might be an upstream regulator of *tbx2* expression. Our preliminary data indicate that loss of BMP signaling also leads to a loss of *tbx3* expression (data not shown), suggesting that both genes are regulated by BMP signaling. In line with our results, others have also suggested that *tbx3* expression is dependent on BMP signaling; in the chick, for example, researchers implanted BMP-soaked beads in chick limb buds and found an induction of Tbx3 expression (Tümpel et al., 2002). In mice, whole embryo culture experiments suggest that exogenous BMP4 can induce *tbx3* expression (Behesti et al., 2006). These data and ours provide support for the idea that BMP4 signaling regulates T-box genes, Tbx2 and Tbx3.

We find that Tbx3, like Tbx2, represses the ventral mesodermal genes *brachyury* and *wnt8*; in contrast with Tbx2, however, Tbx3 does not repress the dorsal mesodermal genes *chordin* and *goosecoid*. This interesting result indicates that Tbx2 and Tbx3 might play different roles in the specification of ectoderm. Tbx2 promotes neural fate and represses epidermal fate; the induction of the epidermal marker, *epidermal keratin*, suggests that Tbx3 might play a role in repressing dorsal ectodermal and promote ventral epidermal fate in the ectoderm. These data point to a partially oppositional role for T-box proteins in patterning the ectoderm with Tbx2 promoting dorsal ectoderm and Tbx3 promoting ventral ectoderm, together allowing for the proper formation of the ectoderm. Future studies will require a careful analysis of *tbx2* and *tbx3* expression throughout gastrula and early neurula stages of development.
Chapter VI: Conclusions and future directions:

In this study, we first describe the role of Tbx2, a T-box transcription factor that is essential for the development of ectodermal fate. We have shown that Tbx2 is expressed in the presumptive ectoderm in the *Xenopus* gastrula embryo, that Tbx2 misexpression is sufficient to inhibit mesendoderm formation, and that Tbx2 functions as a transcriptional repressor. We find that loss of Tbx2 leads to improper expression of mesendodermal genes in the ectoderm. In addition, we show that another member of the T-box family, Tbx3, whose expression is also seen in the prospective ectoderm of gastrula stage embryos, appears to play a role in the repression of ventral mesodermal and endodermal genes, and the promotion of ventral ectoderm. These results suggest that T-box proteins play a critical role in inhibition of ectopic germ layer development in the ectoderm, thereby contributing to the establishment of the early vertebrate body plan.

In the three-tiered blastula embryo, the vegetal pole gives rise to the cells of the endoderm, the marginal zone cells differentiate into the mesoderm and the animal pole cells form the various derivatives of the ectoderm. The yolky cells of the vegetal hemisphere are the source of signals that induce the overlying equatorial cells to form the mesoderm; these signals include TGFβ, Xnrs 1, 2, 4, 5, 6 and Derriere. These signals are essential for both the induction of mesoderm and maintenance of the endoderm (Heasman, 1997). It is generally understood that the animal pole becomes ectoderm due to its location, which is farthest away from the influence of the
endoderm. Due to its location, the endodermally-derived signals and factors that the animal pole are exposed to is limited; in the absence of these signals, the animal pole cells differentiate as ectoderm. However, studies from our lab and others suggest that the animal pole actually plays an active role in its specification. The functional analysis of maternal factors including Coco, Ectodermin, SRF, and the zygotic factors XFDL156, Xema and, from our studies, Tbx2 and Tbx3 indicate that the presumptive ectoderm plays an active role in the repression of mesendoderm (Dupont et al., 2005; Yun et al., 2007; Bell et al., 2003; Sasai et al., 2008; Suri et al., 2005). A major question that still needs to be addressed is why repression of mesodermal and endodermal genes is necessary, when the mesendoderm inducing signals are thought to be excluded from the animal pole. The mere presence of Tbx2, Tbx3 and these other factors suggests that there are mesendoderm-inducing signals, or signal transducers, in the animal pole that must be repressed, or that there is some manner of intrinsic bias toward development of the mesendoderm. The source of these signals and/or the mechanisms underlying this bias have yet to be determined.

Other factors may also play a role in the potential for animal pole cells to form mesendodermal fates. Wardle and Smith have suggested that early gastrula stage embryos contain individual cells called “rogue” cells that express markers outside of their canonical domain (Wardle and Smith, 2004). The dorsal cell marker, goosecoid for example, was initially observed in some ventral cells of the embryo, while the ventral mesodermal marker, Xwnt8, was observed in vegetal and dorsal cells (Wardle and Smith, 2004) suggesting that the cells from early gastrula stage are
initially not fully committed to their respective germ layers. It is possible that ectodermal factors suppress these rogue cells until later stages of gastrulation when the cells become more committed to a particular germ layer and gene expression is representative of their respective germ layer. The identification of mechanism/s underlying proper formation of ectoderm is crucial to our understanding of development.

One way to identify the source of the mesendodermal signal, if one exists, is to determine the common factor that the mesendodermal suppressors of ectoderm act upon. One such factor may be the Tgfβ/Nodal family mediator Smad2. Ectodermin, for example, functions as a SMAD4-mono-ubiquitin ligase that inhibits SMAD2-SMAD4 complex formation and disrupts its binding to the chromatin (Dupont et al., 2005; Xie et al., 2014). Xenopus serum response factor (SRF), a MADS box-containing transcription factor is also important in establishing the germ layers during early development. SRF functions to suppress mesoderm induction caused by Activin/Nodal signals in animal caps by interacting with the Smad2-FAST-1 complex induced by Activin, thereby blocking its function in the nucleus (Yun et al., 2007). The loss of SRF in animal caps has been shown to induce mesendodermal genes (Yun et al., 2007). Other factors involved in ectodermal specification indirectly inhibit Smads and therefore Tgfβ/Nodal signaling. The Zinc finger protein, XFDL156, for example, is important for the specification of ectoderm. This zygotic factor suppresses mesoderm differentiation in the presumptive ectoderm by interacting with the C-terminal regulatory region of the tumor suppressor gene p53, which has been shown to bind to promoters of mesodermal genes and cooperate with Smad2 to form Smad-
DNA complexes for mesodermal differentiation. (Cordenonsi et al., 2003; Sasai et al., 2008; Takebayashi-Suzuki et al., 2003). Sasai et al. has shown that XFDL156 interacts with the C-terminus of p53, thereby inhibiting its interaction with Smad2, thus leading to inhibition of mesoderm in the ectoderm (Sasai et al., 2008). These data point to active suppression of Smad2 in the ectoderm (Daniels et al., 2004), suggesting that upon loss of ectodermal specification factors, Smad2 might be a key factor in the ectopic formation of mesendoderm in the animal cap. Indeed, studies have shown that the induction of different cell fates by Activin in the animal cap assay can be recapitulated by injection of increasing amounts of Smad2 transcript (Graff et al., 1996). It will be interesting to identify whether Tbx2 also functions to repress mesendoderm by interacting with Smad2; to this end, we plan to undertake experiments in which we will attempt to co-immunoprecipitate Tbx2 and Smad2. Eomesodermin, another T-box protein important in the formation of mesoderm, has been shown to form a complex with phosphorylated Smad2 to activate several mesodermal genes (Picozzi et al., 2009). To test whether Tbx2 can also interact with Smad2, we recently constructed a Myc-tagged version of Tbx2 whose function in mesendoderm repression assays in indistinguishable from that of native Tbx2 (data not shown). If Tbx2 is able to interact with Smad2, it would lend support to a model in which Smad2 functions as a major target for factors required for the suppression of extra-ectodermal fate.

It will be interesting to determine which, if any, of the ectodermal pathways converge on Tbx2 or Tbx3. In other words, can Tbx2 or Tbx3 substitute for the loss of Xema or XFDL156 or vice versa? Spatial expression data suggests that xfdl156, xema, tbx3 and tbx2 (Sasai et al., 2008; Suri
et al., 2005) begin expression around stage 8 with a stronger signal apparent at late blastula stages. It will be interesting to determine whether any of these genes are functionally redundant, especially since they seem to begin their expression around the same stages. Can any of the genes make up for the loss of the others? If so, it raises more interesting questions, including why the embryo needs to express so many different genes to suppress mesendoderm. These factors may have different, as yet, uncharacterized roles beyond repression of mesendoderm as the embryo develops. To begin to address these issues, we have recently obtained constructs for all of the above-mentioned zygotically-expressed factors; we plan to perform epistasis experiments in which we will co-inject Tbx2 morpholino and RNA from xema, xfdl156, or tbx3, and assay for mesendoderm suppression.

Our studies indicate that Tbx2 represses BMP4 activity and epidermal gene expression, while promoting neural ectoderm. However, tbx2 appears to be expressed throughout the animal pole ectoderm, including the ventral ectoderm, at least at mid-gastrula stages. While we need to perform further studies, the data obtained so far suggests that Tbx2 and Tbx3 may have dual functions in the ectoderm, with Tbx3 promoting ventral ectoderm and Tbx2 promoting neural ectoderm. To further analyze this, we will need to perform in-situ hybridization to carefully observe dorsal versus ventral differences in Tbx2 and 3 expression patterns. The results from these studies may prove to be very interesting as they would help to delineate and understand the function of ectodermal T-box proteins during early development.
Our data demonstrate that Tbx2 is necessary and sufficient for the suppression of ectopic germ layer formation in the presumptive ectoderm. Our data also indicate that Tbx2 may mediate this function in part through direct repression of Bix4, a known target in the mesoderm and endoderm of the T-box proteins Brachyury and VegT. However, there may be additional genes, including members of the closely related Mix family genes (Mead et al., 1998; Tada et al., 1998) that are suppressed by Tbx2 in the presumptive ectoderm. Future endeavors will therefore include isolating additional Tbx2 targets to identify other downstream effectors and pathways that mediate Tbx2 function.
References:


